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In vitro and in vivo studies of toxic shock syndrome toxin-1 and tampon associated fibers.

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IN VITRO AND IN VIVO STUDIES OF TOXIC SHOCK SYNDROME TOXIN-1 AND TAMPON ASSOCIATED FIBERS

A Dissertation Presented

By

MOLLY PICKETT

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ABSTRACT

In Vitro and *In Vivo* Studies of Toxic Shock Syndrome Toxin-1 and Tampon Associated Fibers

September 1987

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The involvement of tampons in development of toxic shock syndrome (TSS) was investigated in three stages. First, toxic shock syndrome toxin-1 (TSST-1) was isolated from a known TSST-1 producing strain of *Staphylococcus aureus*. Iodinated toxin was identified by reaction with specific anti-TSST-1 antibody and resolution of the antibody-adsorbed protein by SDS-PAGE and autoradiography. Additional toxin was purified by ion exchange chromatography on CM sephadex C25 or Rexyn 102 cation exchange resins followed by molecular exclusion chromatography. Each method yielded a purified toxin with a characteristic molecular weight of 22,000 daltons and a pI of 7.0. Purified TSST-1 was used to produce specific antiserum in a goat. Resulting immune IgG was used as a ligand for affinity purification of toxin.

Secondly, production of TSST-1 in the presence of tampon fibers and magnesium (Mg^{++}) was studied *in vitro*. Polyester foam (PEF, Rely tampon) and polyacrylate rayon

(PAR), two fibers more frequently associated with TSS stimulated TSST-1 production while cotton and rayon did not. The influence of Mg^{++} appears to be different on PEF and PAR, PAR chelated Mg^{++} from aqueous solutions and brain heart infusion broth (BHI) while PEF did not. Addition of Mg^{++} had a direct effect on toxin production in the presence of PAR and had a modulating effect in PEF/BHI cultures. Lack of aeration decreased the amount of toxin produced in the presence of PEF but had no effect on toxin produced with PAR or cotton.

Finally, intravaginal inoculation of guinea pigs with TSS associated strain MN-8 and PEF or cotton induced comparable levels of TSST-1 production in contrast to *in vitro*. Only the animals that had been implanted with PEF developed a serum antibody response to TSST-1 as measured by RIA and confirmed by immunoblot. This indicates a fiber specific enhancement of the humoral response and may have implications as to the effect of certain fibers on the development of toxic shock syndrome.

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LITERATURE REVIEW

Description of the organism. The association of gram positive cocci with disease has long been recognized in the history of microbiology. Not only could these organisms cause infection invasively, they also had the ability to produce certain "poisonous" substances which were toxic in their own right. Cell-free culture supernatants from these organisms could produce inflammation, necrosis, or death in laboratory animals. With this finding, came the understanding that the organisms could express a multiplicity of toxins causing a variety of effects (Elek,1959)

S. aureus along with other Micrococcaceae is indigenous to humans and other mammals living for the most part as a normal inhabitant of the skin, upper respiratory and intestinal tracts. The association of plasma clotting ability with pathogenicity has resulted in division of the species by the ability to produce coagulase. All coagulase positive staphylococci are identified as Staphylococcus aureus (Davis et al, 1980).

Staphylococcus aureus grows under aerobic and anaerobic conditions, although more prolifically under aerobiosis. These organisms are nonmotile and divide in more than one plane, resulting in grape-like clusters of bacteria. This

species is the most resistant of the non sporeformers to adverse environments and is resistant to heat, desiccation, high salt concentrations, and disinfectants. The ability of S. aureus to acquire resistance to new antibiotics via bacteriophage also contributes to its success and prevalence as a pathogen (Davis et al., 1980. Dubos, 1958).

In addition to potential for causing suppurative diseases in any organ of the body, S. aureus is the etiological agent for a number of toxinoses including staphylococcal food poisoning, pseudomembranous enterocolitis, exfoliative skin disease and the more recently recognized toxic shock syndrome (TSS). While staphylococcal infections and diseases have been the subject of numerable studies to date, the mechanism of how the ubiquitous S. aureus transcends its commensal relationship into one of pathogenesis remains to be explained.

In addition to most mentrually associated TSS strains producing a specific marker toxin termed toxic shock syndrome toxin-1 (TSST-1), these organisms have certain other distinct phenotypic characteristics. Todd et al. (1984) examined the phenotypes of isolates from TSS patients and found them significantly different from control strains for the following characteristics: decreased hemolysis, absence of plasmids, bacteriocin susceptibility, less susceptible to arsenate, pigment production, and proteolysis of casein. Proteolysis and

production of TSST-1 accounted statistically for all other phenotype variations between TSS associated and controls strains of S. aureus. Chu et al. (1985) also found a high correlation between tryptophan auxotrophy and the ability to produce TSST-1.

Altemeir et al. (1983) reported that the majority of TSS associated strains of S. aureus were in phage group I and typable with the 29/52 phages. Many were untypable and 5 new phages were isolated reducing the percent of untypable strains.

History of toxic shock syndrome. Although toxic shock syndrome was not recognized as a discrete clinical entity until 1978 (Todd et al.) there have been previous reports of illness resembling TSS that were associated with S. aureus as early as 1927 (Stevens, 1927, Aranow and Wood, 1942. Dunnet,1960). The Bundaberg disaster in Australia, where children were fatally inoculated with a S. aureus contaminated vaccine may be attributable to TSS (Chesney et al.,1984).

Todd and coworkers' report (1978) described seven children, both male and female stricken with severe multi-system illness and symptoms including fever, hypotension, headache, confusion, rash, vomiting, diarrhea, oliguria and subsequent peeling of skin on the palms and soles. Staphylococcus aureus phage type 1 was isolated from

various sites of five patients including mucosal surfaces, lungs, and abscesses.

In 1980, through epidemiological studies of increasing numbers of TSS cases (Shands et al.,1980. Davis et al.,1980), the association of TSS with menstruation and tampon use was recognized. By September of the same year, while many brands of tampons were associated with TSS, it was found that women using the Rely tampon (Procter and Gamble Co) were at greater risk for development of the syndrome (CDC,1980. Schlech et al.,1980). The manufacturer of Rely immediately withdrew the product from the market.

As epidemiological studies were continued, it was recognized that at least 90 % of women afflicted with menstrual TSS were colonized intravaginally with S. aureus (Davis et al., 1980. Shands et al.,1980). A high percentage of the vaginal isolates had the ability to produce marker proteins then identified as staphylococcal enterotoxin F (SEF) and pyrogenic exotoxin C (PEC, Bergdoll et al,1981. Schlievert et al,1981).

By 1982, of the TSS cases that were reported, 10 to 20 % were males or nonmenstruating females with staphylococcal infections identified at other body sites (Reingold et al.,1982). Nonmenstrual TSS can occur in any situation where there is a disruption of the skin or mucous membranes allowing a localized infection of S. aureus to develop and

elaborate toxins (Friedell and Mercer, 1986).

Bonventre and colleagues (1983) systematically compared PEC and SEF and found the two toxins to be identical. As a result of this finding it was agreed that the two toxins were the same and would be termed toxic shock syndrome toxin-1 to allow for involvement of other unidentified toxins in the development of the syndrome.

Association of toxins with TSS. Todd et al. (1978) in the first article describing TSS as a discrete clinical entity, found a toxin associated with isolates from 5 patients that produced a positive Nikolsky sign in newborn mice. The toxin was distinct from staphylococcal exfoliatin and produced epidermal cleavage at a higher plane.

Kapral (1981) isolated a toxin believed to be the same as that isolated by Todd and coworkers. The toxin required the presence of yeast extract in growth medium, was stable in acid and could be degraded by trypsin. This toxin was heat labile and antigenically distinct from exfoliatin. It was suggested that this toxin might be responsible for the desquamation associated with TSS although the author stated that there was no clear evidence to support this.

While it was known that TSS isolates could produce one or more toxins no specific toxin was consistently found in early investigations (Davis et al., 1980). There was no

recognized clinical markers for TSS until the report of Cohen and Falkow (1981). These researchers found 2 proteins with molecular weights of 30 and 33 kDa that were produced by 78% of TSS isolates but only 25% of controls. There was no biological activity attributed to these proteins.

Later in 1981, two separate laboratories reported isolation of a toxin that was associated with strains of Staphylococcus aureus isolated from patients with TSS but not with control strains (Schlievert et al., 1981 and Bergdoll et al., 1981). Schlievert's group reported that 100% of TSS associated strains produced the toxin while only 16% of control strains did. They designated the toxin staphylococcal pyrogenic exotoxin C (PEC) because it produced fever in rabbits after iv injection. The toxin was also found to enhance lethal endotoxin shock when coadministered with endotoxin isolated from Salmonella typhimurium. PEC was purified by differential precipitation with ethanol and thin-layer isoelectric focusing. It was found to have an isoelectric point of 7.2 and a molecular weight of 22 kDa. The purified toxin was reported to have non-specific mitogenicity using rabbit splenocytes and human peripheral blood lymphocytes enriched for T cells. PEC did not cause stimulation of preparations enriched for B cells indicating that the mitogenicity was T cell specific. PEC suppressed the IgM antibody response of

murine splenocytes to erythrocytes.

Bergdoll and coworkers (1981) reported that 61 of 65 strains of S. aureus produced an enterotoxin like protein and named it staphylococcal enterotoxin F (SEF). In contrast only 4.6% of non TSS strains were found to produce this protein. SEF was purified by ion exchange chromatography with CG-50 ion-exchange resin and CM-Sepharose followed by gel filtration on sephacryl S-200. It was found to have a molecular weight of 20 kDa, an isoelectric point of 6.8 and was resistent to digestion with trypsin but slowly degraded by pepsin at pH 4.5. The purified toxin produced emesis and diarrhea in monkeys as a result of intragastric injection.

Barbour (1981) compared 15 vaginal isolates of S. aureus from TSS patients to 18 non TSS associated vaginal isolates. The phenotypic traits that were more frequent in the TSS isolates included arsenate resistance, hemoglobin proteolysis and surprisingly a lack of lethality of culture filtrates to injected rabbits and chicken embryos. A protein with a molecular weight of 22 kDa and a pI of 7.2 was found in culture filtrates from all TSS isolates. This was thought to be the same protein reported by Schlievert et al.(1981).

While the characterizations of both PEC and SEF and their high prevalence in staphylococci isolated from TSS patients were similar the proteins were treated as two distinct

toxins. Bonventre and collaborators (1983) compared SEF and PEC production by TSS associated isolates in two separate laboratories and found 100% concordance. Not one isolate was found to produce only one of the two toxins. They also found that purified PEC and SEF migrated with the same mobilities by SDS-PAGE under both reducing and non reducing conditions and had identical isoelectric points of 6.8-6.9. Specific antisera prepared against PEC or SEF reacted equally well with either protein when analyzed by double immunodiffusion or western blotting. It was concluded that SEF and PEC were probably the same TSS associated staphylococcal toxin.

Reeves et al (1984) used two techniques to purify TSST-1, preparative isoelectric focusing and chromatofocusing and found that toxin purified by the second method was more homogeneous. Three molecular weight variants of the toxin were found by immunoblotting with a monoclonal anti TSST-1 antibody as the probe. The molecular weights of the variants were 21.4, 22.1 and 23.2 kDA by SDS-PAGE. However the molecular weight that was calculated with the Stokes - Einstein equation based on sucrose gradient centrifugation and gel filtration data on non denatured TSST-1 was 18.9 kDa. Control and TSS strains of S. aureus were analyzed for their ability to produce TSST-1 using isoelectric focusing and double immunodiffusion. The isoelectric focusing procedure was found to over estimate the TSST-1

positive strains in comparison to immunodiffusion. By immunodiffusion, TSST-1 was found to be present in 100% of the vaginal TSS strains and 62% of the non-vaginal isolates. The control non-TSS strains were positive for TSST-1 in 16% of the vaginal isolates and 23% of the non-vaginal strains.

Chow et al (1984) examined the vaginal colonization of healthy women for vaginal colonization with TSST-1 positive strains of Staphylococcus aureus. S. aureus was isolated from 6.8% of the subjects and toxigenic strains from 2.6%. A similar degree of vaginal S. aureus colonization was also found by Linnemann and coworkers (1982). Production of TSST-1 was determined by analytical isoelectric focusing of culture supernatants concentrated by ethanol precipitation. Coisolation of E. coli and previous history of a urinary tract infection were the only factors that were significantly associated with the presence of a toxigenic strain. Other factors that were examined but not found significantly associated were phase of the menstrual cycle, contraception, tampon use or brand, sexual activity or genital symptoms. The probability of coisolation of E. coli with a TSST-1 strain was 8 to 11 times higher than with a non-toxigenic strain or from women who were not colonized with staph. The authors suggest that the dual colonization of these two organisms may lend support to the theory that the vaginal E. coli and associated endotoxin

may contribute to the pathogenesis of TSS.

Garbe et al (1985) isolated strains of S. aureus from normally sterile sites on patients with nonmenstrual TSS and evaluated them for the production of TSST-1. They found that only 62% of the nonmenstrual TSS isolates produced the toxin while 93% of the menstrual TSS isolates did. The TSST-1 negative strains were more likely to be associated with a fatal outcome of the disease than TSST-1 positive strains. The TSST-1 negative strains were further evaluated by infecting a subcutaneous infection chamber in rabbits. Sixty percent of the rabbits developed some of the symptoms of TSS and 9 out of 25 rabbits died. The authors postulated that these results indicate the role of other unidentified toxins in TSS and that the ability of S. aureus to produce TSST-1 may not be a requirement for causation of the disease.

Schlievert (1986) confirmed that fewer nonmenstrual TSS isolates were TSST-1 producers than menstrual TSS isolates. Thirty nonmenstrual TSS isolates were examined for the ability to produce other enterotoxins or exfoliative toxins. It was found that 78% of the nonmenstrual strains produced either TSST-1 or enterotoxin B as compared to 20% of non-TSS/nonmenstrual isolates. Enterotoxin B was found in strains that did not produce TSST-1. This toxin was rarely found associated with vaginal TSS isolates. Since enterotoxin B has many of the same biological properties of

TSST-1 and the streptococcal pyrogenic exotoxins and streptococcal scarlet fever has similarities to TSS it was suggested that this toxin may play a role in the pathogenesis of the disease.

Crass and Bergdoll (1986) studied sera and S. aureus isolates from patients with probable or confirmed TSS. TSST-1 was produced by 91.6% of the isolates alone or with other enterotoxins. These investigators found as did Schlievert (1986) that enterotoxin B was not produced in combination with TSST-1 by any of the isolates. Strains that produced TSST-1 and enterotoxin C or only enterotoxin B were found in significantly more nonmenstrual than menstrual isolates. The serum antibody titers of patients with TSS and a control group were compared. Most of the patient with TSS did not have detectable antibody titers to TSST-1 (82.4%) while 77.7% of the controls had titers of at least 1/800. Sera from TSS patients also had low antibody against enterotoxins A, B and C. The authors concluded that this may be indicative of an immune deficiency which inhibits the production of antibodies against TSST-1 and other staphylococcal enterotoxins and thus renders these subjects more susceptible to development of TSS.

Another report by Crass and Bergdoll (1986) presented evidence that other enterotoxins besides TSST-1 could be implicated in TSS particularly enterotoxin B. Animal studies have shown that enterotoxin B can induce many of

the symptoms of TSS and it was not found in any strain that is able to produce TSST-1. Some of the patients followed during the course of TSS developed antibodies against enterotoxin B with no change in anti-TSST-1 titer indicating that enterotoxin B but not TSST-1 was produced during the disease.

A survey of multiple body sites for the prevalence of S. aureus in normal women was made by Lansdell and coworkers (1984). These researchers isolated S. aureus from external genitalia in 26% of the women tested. Of these subjects, half were also positive for S. aureus intravaginally. One fifth of the genital isolates were positive for TSST-1. Thirty percent of the women were culture positive for S. aureus isolated from nasal sites. A positive nasal culture was not found to be predictive of colonization of the labia or vagina. The authors surmised that nasal carriage did not influence genital colonization but vaginal intrusion probably by fingers may be responsible for introduction of S. aureus.

A retrospective analysis for the presence of TSST-1 in S. aureus isolated from 1956-1982 was conducted by Hayes et al (1984). The presence of TSST-1 was confirmed by isoelectric focusing and solid-phase radioimmunoassay. A 15% false positive rate by isoelectric focusing (IEF) as confirmed by the radioimmunoassay was noted. It had been postulated previously (Broome et al., 1982, Kass, 1982,

Reingold et al., 1982) that the increase in cases of TSS which occurred in 1978 was due partly to an increase in TSST-1 positive strains. Hayes and coworkers found that although there were temporal changes in the prevalence of TSST-1 positive strains, these did not correlate to the increased incidence of TSS. The first TSST-1 positive strain from the group tested was isolated in 1957. The peak prevalence of TSST-1 positive strains was found in 1976 isolates, four years before the peak incidence of TSS. There was also no difference in rate of TSST-1 positives strains according to the body site where it was isolated or by the geographic location.

Nasal carriage of toxin producing strains of S. aureus and prevalence of serum antibody to TSST-1 was investigated by Jacobson et al. (1986). The rate of nasal carriage was 35%. This is similar to other reports (Ritz et al., 1984, Martin et al., 1982, Lansdell et al., 1984). The carriage rate of TSST-1 producing strains was 7.2%. Serum antibody titers of 1/100 or greater were found in 90% of the patients tested who were greater than 30 years old. About two thirds of the patients who were thirty or under had comparable antibody titers. Only one out of 2000 patients tested was found to have a combination of colonization with a TSST-1 producing strain with low antibody titer to the toxin. Even though the patient had surgery at the colonized site TSS did not occur. The authors suggest that

this indicates other factors are involved in development of the disease.

Although the ability to produce TSST-1 has been primarily associated with coagulase positive strains of S. aureus, Crass and Bergdoll (1986) have presented evidence for production of the toxin by coagulase negative strains. Coagulase negative and TSST-1 positive strains were isolated from 7 patients with TSS. In 2 of the patients coagulase positive strains were coisolated but these were not found to be toxigenic. It was suggested that in cases where coagulase positive strains are not found, coagulase negative strains may be the causative agents of the disease.

The colonization of body surfaces by specific microorganisms may be enhanced by the production of substances which are inhibitory to other bacteria. Peters et al. (1985) have reported that vaginal strains of S. aureus produce at least three inhibitory substances, and one of these is more frequently associated with TSS strains. These bacteriocin-like substances were found to have activity against alpha-hemolytic streptococci, and lactobacilli but not other staphylococci, enterobacteriaceae or enterococci by the deferred antagonism test. The inhibitory activity against certain vaginal microflora may have a role in the development of TSS.

Todd et al (1985) reported that TSS associated strains of S. aureus are more proteolytic than non TSS strains particularly in the presence of EDTA, cysteine and 5% CO_2. These proteolytic enzymes may contribute to the pathogenesis of TSS by altering staphylococcal or human proteins resulting in activation or enhanced biological activity.

Biologic function of TSST-1. Many of the metabolic responses to infection and inflammation are thought to be mediated by interleukin-1 (IL-1, Sobrado et al., 1983). Ikejima et al. (1984) found that culture supernatants of certain TSST-1 producing strains of S. aureus induced production of human interleukin-1 while control culture supernatants from non TSS related strains did not. The induction of IL-1 from human monocytes was determined by in vivo induction of fever in rabbits injected with supernatants from monocyte cultures culture supernatants from TSS associated strains. In vitro assays demonstrated that the toxin was mitogenic to thymocytes. These effects were associated with one specific protein with a molecular weight of 30 kDa. The purified toxin was found to be a more potent inducer of IL-1 than endotoxin. These investigators postulated that the induction of IL-1 may be intrinsic to the pathogenesis of TSS.

Parsonnet et al. (1985) confirmed that highly purified

TSST-1 was a potent inducer of IL-1. Induction of IL-1 induction could be inhibited by hydroxycortisone but not indomethacin, two antiinflammatory agents. The authors suggested that IL-1 induction may be an explanation for the high fever that is invariably present in TSS.

It has been found that while most isolates from menstrually associated cases of TSS elaborate TSST-1 (Bergdoll et al., 1981, Schlievert et al.,1981), a significant portion of nonmenstrual cases do not (Reeves et al., 1984. Garbe et al., 1985). Although TSST-1 appears to be intrinsically involved in the pathogenesis of the disease as demonstrated by de Azavedo et al. (1985) using a cloned TSST-1 gene in a rabbit uterine model, it is not a requisite for elaboration of the disease. Parsonnet and coworkers (1986) examined the induction of IL-1 by nonmenstrual strains of S. aureus with a view towards confirming a role for IL-1 induction in the pathogenesis of TSS. They found that all of the nonmenstrual TSS isolates did induce IL-1 production whether or not they were able to produce TSST-1. Although the TSST-1 producing strains were found to be more potent inducers of IL-1 than the non-TSST-1 producing strains, the non-producing strains were significantly more potent IL-1 inducers than staphylococcal strains isolated from other clinical isolates. Since many of the criteria for TSS including fever, disrupted electrolyte balance and kidney, liver, muscle dysfunction

are attributable to properties of IL-1 (Dinarello, 1984), the finding that all TSS associated strains tested, both menstrual and nonmenstrual induce IL-1 production provides additional support for its involvement in TSS.

In addition to its properties as a thymocyte mitogen and IL-1 inducer, Micusan et al (1986) have presented evidence that TSST-1 induces interleukin 2 (IL-2) production in human peripheral blood and murine splenic lymphocytes. Incubation of TSST-1 with either of these two cell preparations resulted in the production of a soluble factor that was strongly mitogenic to IL-2 dependent indicator cells. The authors concluded that the IL-2 inducing capacity was due to the intrinsic mitogenicity of TSST-1, not because of immune reactions against the toxin as an antigen.

Other investigators (Poindexter and Schlievert, 1985) demonstrated that lymphocytes from cord blood responded to TSST-1 as a mitogen and concluded that previous exposure was not a requirement for this response. This also indicates a role for TSST-1 as an immunomodulating molecule.

Uchiyama et al (1986) investigated the proliferative response and IL-2 production by TSST-1 stimulated T cells. They found that TSST-1 is mitogenic for mature T cells from both thymic and peripheral lymphoid tissue but not for immature T cells or IL-2 dependent cells such as cytotoxic

T lymphocytes. The addition of adherent macrophages or a IL-1 containing macrophage lysate to TSST-1 stimulated T cell preparations significantly enhanced IL-2 production in comparison to control T cell preparations. The authors suggest that IL-2 production by TSST-1 stimulated lymphocytes requires the presence of Ia positive accessory cells. It was postulated that overproduction of IL-2 and perhaps other lymphokines in TSS afflicted patients may be a factor which accelerates or exacerbates the development of TSS.

Host defense mechanisms. Animal studies. Because chicken embryos are susceptible to lipopolysaccharide (LPS) the effect of TSST-1 in combination with LPS was evaluated by De Azavedo and coworkers (1985). Similar to the effect noted in rabbits (Schlievert, 1982, Schlievert et al., 1981) TSST-1 increased the lethal effect of LPS although it had no effect by itself. The LD_{50} in the presence of sublethal doses of LPS was found to be TSST-1 dose related, with the greatest lethality occurring at 1 and 10 ug of TSST-1. Surprisingly at 100 ug TSST-1 or greater no enhanced lethality was observed.

Injection of TSST-1 into laboratory rodents including mice, hamsters, rats, and guinea pigs, has not been shown to have a toxic effect (Quimby and Nguyen,1985). Melish et

al. (1982) detected TSST-1 in abscesses on mice inoculated with TSS associated strains of S. aureus only 2 hr after inoculation and detected the toxin in serum beginning at four hr after inoculation and peaking at 24 hr.

A synergistic lethal effect was observed in mice coinoculated with both S. aureus and Candida albicans by Carlson (1983). TSST-1 producing strains that did not produce other toxins were the least virulent when injected alone but among the most lethal when injected in combination with C. albicans. This led the author to conclude that TSST-1 is probably toxic to mice under certain conditions, although influence by hemolysins could not be ruled out. It was postulated that the influence of the yeast may be to inhibit phagocytosis which in turn would impede elimination of endotoxins. The effect of elevated endotoxin levels may then be amplified by TSST-1. A number of similar symptoms were noted for the dually infected mice and TSS patients including conjunctivitis, circulatory, gastrointestinal and neurological abnormalities, and a rash with subsequent desquamation.

Tierno et al. (1987) inoculated mice intraperitoneally with supernatants from cultures of E. coli and TSS associated strains of S. aureus grown in the presence of a variety of tampons. Inoculation of culture supernatants from Rely, Playtex super plus and Tampax super plus resulted in the death of all animals within 48 hr.

Supernatants from other tampon cultures including OB super, Pursettes super plus, and Kotex super resulted in death of 5 out of 15 animals at 72 hr. Control inoculations of supernatants from a non-TSST-1 producing strain of S. aureus and E. coli grown in a Rely tampon or a TSST-1 producing strain grown in a cotton tampon did not induce mortality. Leachable compounds from Rely tampons in combination with a crude exotoxin preparation from TSS associated S. aureus and endotoxin also produced mortality in all animals inoculated. It was concluded that certain tampons provide necessary conditions for production of exoproteins from S. aureus which in combination with endotoxin are lethal.

TSST-1 injection in rabbits can induce certain symptoms common to TSS in humans including: conjunctivitis, fever, respiratory distress and sometimes diarrhea (De Azavedo et al. ,1983). Higher doses of TSST-1 can result in death preceded by vascular collapse, renal failure and labored breathing (Schlievert, 1982). Notermanns and Dufrenne (1982) reported the LD_{50} for rabbits to be 6 and 180 ug TSST-1 by subcutaneous and intravenous injection respectively.

Much variability has been reported in the response of rabbits to TSST-1 (Reeves et al., 1986, Quimby and Nguyen, 1984). Variability in response has been shown in different strains, sexes and ages of rabbit, and is also attributable

to the inoculation procedure.

An artificial infection chamber produced by implanting a perforated polyethylene ball subcutaneously in rabbits has been used as a model system to study TSS. Best et al. (1984) found that rabbits immunized with either acidic or neutral proteins from TSS isolates were not as susceptible to mortality when infected in a subcutaneous chamber. Male rabbits in this study were more susceptible to lethal infection than female rabbits but by castration could be made equally susceptible. Consistent with the findings of Best and colleagues, Scott et al. (1986) used purified TSST-1 to immunize rabbits before inoculation of infection chambers and none of the rabbits that were preimmunized succumbed to infection while one half of the rabbits without prior immunization died. In an earlier study using the same model system, Scott et al. (1983) found that 11 out of 15 TSS associated strains induced lethality while none of 5 control strains did.

Best et al., (1986) found that estrogen treatment of male rabbits infected with a TSS isolate reduced mortality and prolonged survival while androgens and progesterone had no effect on the susceptibility of female rabbits. Reeves and coworkers (1986) purified TSST-1 by chromatofocusing and affinity chromatography and found that both male and female New Zealand white rabbits were susceptible and that the susceptibility increased with age. Simultaneous

injection of endotoxin and TSST-1 did not increase lethality although injection of endotoxin iv after subcutaneous injection of TSST-1 did. Animals that died after injection with TSST-1 had extensive damage to the thymus, spleen, liver, and lymph nodes. The pathology noted was comparable to that observed in human victims of TSS and in rabbits inoculated in subcutaneous infection chambers with TSST-1 producing strains of S. aureus in subcutaneous infection chambers.

De Azavedo et al. (1985) used an intrauterine diffusion chamber in rabbits inoculated with TSST-1 positive and negative strains. All of the rabbits inoculated with the TSST-1 positive strain had symptoms and 6 out of 15 died. None of the TSST-1 negative strain inoculated rabbits were effected. This experiment showed that the toxin is produced in vivo and can traverse the uterine epithelial cells into circulation. A TSST-1 negative strain of S. aureus was used as a host for a plasmid carrying the cloned gene for TSST-1 and caused identical symptoms in rabbits as the TSST-1 positive strains implanted in the intrauterine chamber. It was concluded that TSST-1 clearly has a role in the development of TSS.

The initial study by Bergdoll et al. (1981) showed that TSST-1 caused emesis and diarrhea after intragastric administration in cynomologus monkeys. Later studies with both cynomologus and baboons failed to confirm this effect

(Reiser et al., 1983). Pollack et al. (1983) introduced 6 TSST-1 producing isolates of S. aureus into the vaginas of rhesus monkeys along with a modified Rely tampon. None of the animals developed any symptoms of TSS although all monkeys were colonized with S. aureus. Four monkeys developed high titers to TSST-1. In subsequent cycles, the animals were inoculated with addition TSST-1 and still failed to elicit any response. Quimby and Nguyen (1984) found that injection of purified TSST-1 into baboons produced many of the symptoms of TSS with the exception of desquamation.

Involvement of endotoxins in TSS. A synergistic effect of endotoxin and TSST-1 can be demonstrated in rabbits and has been proposed as a possible mechanism in the development of TSS (Stone and Schlievert, 1987). Pasteurella multocida free and normal rabbits differed in their response to TSST-1. A low incidence of mortality was found when TSST-1 was administered to the pathogen free rabbits in comparison to the controls. All of the control rabbits had detectable levels of circulating endotoxin before administration of the TSST-1 while there was none detected in the pathogen free rabbits. All rabbits had detectable levels of endogenous endotoxin after administration of TSST-1. Polymyxin B an antibiotic effective against endotoxin was found to significantly reduce mortality when control

rabbits were pretreated prior to administration of TSST-1. Acute and convalescent phase sera from TSS patients was also analyzed for endotoxin and was found only in the acute phase samples. These authors proposed that since many of the clinical features of TSS are common to those of endotoxin shock, a possible mechanism for TSS is toxin mediated inhibition of hepatic endotoxin clearance.

The variability of response by rabbits to TSST-1 may be attributed, at least in part to the presence of circulating endotoxin. Schlievert (1982, 1983) has reported that tetracycline treated rabbits are more resistant to TSST-1 toxicity. In this study, treatment of rabbits with TSST-1 was found to enhance the susceptibility to lethal endotoxin shock by as much as 50,000 times. Other investigators (Peterson et al., 1983) found that rabbits could be protected by the injection of methylprednisone and IgG before dosage of TSST-1. Rabbits more than two years old were found more susceptible to TSST-1 than young rabbits by De Azavedo and Arbuthnott (1984) and male rabbits were found by Best et al. (1984) to be have enhanced susceptibility to infection with a TSS associated strain of S. aureus over female rabbits.

Fujikawa et al. (1986) described a decrease in clearance of exogenous endotoxin in rabbits treated with TSST-1 which suggests that TSST-1 may inhibit endotoxin removal from the system. Schlievert (1983) suggest that the increased

susceptibility to endotoxin in the presence of TSST-1 could be caused by any of several factors including a suppression of the humoral antibody response or decreased function of the reticuloendothelial system. The observation that the respiratory systems of TSS patients are often colonized with gram-negative organisms is consistent with the possible differential stimulation of T suppressor cells but not T helper cells by TSST-1. Correspondingly, phagocytosis of red blood cells noted in victims of TSS may be a result of macrophage activation by TSST-1-stimulated delayed hypersensitivity T cells.

Changes in neutrophil function. Changes in the bacteriocidal properties of neutrophils from patients recovered from TSS have been documented by Berger et al. (1986). Five women who had recovered from TSS had more menstruation associated decreases in the ability of their neutrophils to kill S. aureus than controls. This may indicate that there is transient menstrually related decrease in neutrophil function which could increase susceptibility of certain women to TSS. The authors suggest that such defects, transient or otherwise, may allow a more rapid proliferation of S. aureus and concomitant release of toxins.

A separate case report (Berger et al., 1986) described a woman who had a persistent neutrophil defect in contrast to

a temporal one. Neutrophil function was tested during the acute phase of TSS and over time after recovery. Interestingly, the neutrophils were most effective at killing bacteria during the acute phase. At this time, the bacteriocidal ability was comparable to controls. Samples taken after recovery had decreased ability to kill the test organism. This suggests that activation of the neutrophils is involved in host response to TSS and reiterates that there may be a causal relationship between neutrophil function and development of TSS.

Development of antibodies to TSST-1. Although there is a high prevalence of antibodies to TSST-1 in the general population (Vergeront et al.,1983), there are decreased or non-detectable levels of these antibodies in TSS patients (Bergdoll et al.,1981, Davis et al.,1982, Stolz et al.,1985). In a study of sera from German women 98% of the samples from subjects over 25 years of age had antibody titers to TSST-1 while 11% of the under 25 age group had none (Loch et al , 1986). Similarly, Jacobson et al (1986) found over 90 % of those tested over 30 had antibody titers of at least 1/100 while about 70 % of the under 30 group had equivalent titers.

Bergdoll et al.(1981) noted that patients with TSS had a low prevalence of serum antibodies to TSST-1 (SEF) in comparison to control sera. These researchers commented

that the low antibody titers in the TSS patients may indicate increased susceptibility. Many of the TSS patients did not have an increase in anti-SEF serum titer after the infection and appear to remain susceptible to recurrences. In a later report, Bergdoll et al. (1982) suggested susceptibility to TSS can be identified by determining the serum titer to SEF. In 8 women with menstrual TSS, Notermans et al. (1983) observed that only 2 developed increased titers in the convalescent phase of the illness. Stolz and coworkers (1985) found that only 9.5 % of confirmed menstrual cases had detectable anti-TSST-1 antibodies in sera sampled in the first seven days of the illness. Thirty-three percent of the patients tested developed titers 7-9 days post onset. In contrast, about 36 % of the nonmenstrual patients had detectable titers to TSST-1 early in the disease. These authors concluded while the presence of anti-TSST-1 antibodies has not been confirmed as protective, their absence does indicate susceptibility. It was also noted that younger patients have a greater rate of seroconversion than older patients perhaps indicating that for many young TSS patients, it is their first exposure to the toxin rather than a specific immune defect.

An effort to associate genetic markers with acute TSS by Latham et al. (1983) was not successful although it was acknowledged that the markers investigated may not have

been appropriate. It was also suggested by Schlievert (1983) that the poor humoral response may be due to a lack of antigenicity of the TSST-1 molecule.

Schlievert (1983) found that of 6 rabbits immunized repeatedly with TSST-1 only 3 developed antibodies. The non- immune rabbits were nevertheless able to elicit a response against sheep red blood cells indicating that the lack of response to TSST-1 was specific and not a general immunodeficiency. These animals did develop a delayed hypersensitivity reaction to TSST-1 inoculated intradermally indicating that the T cells at least were sensitized to the toxin.

Production of TSST-1. Schlievert and Blomster (1983) investigated the effect of culture conditions and medium components on production of TSST-1 (PEC). Assay of the toxin was accomplished by double immunodiffusion using serial dilutions of concentrated cell supernatants. The optimum temperature for the production of TSST-1 was 37°C and 32 times more toxin was produced by aerated cultures than by anaerobic ones. Strain MN-8 was found to produce 8 times more toxin than strain 587 (19.2 ug/ml versus 2.4). Toxin production was affected by pH with minimal toxin produced at pH 6 and maximal amounts of growth and TSST-1 production occurring at pH 7-8. The type of medium also influenced toxin production. Tryptic soy and Todd-Hewitt

media did not enhance toxin production while brain heart infusion broth did. Addition of glucose at concentrations up to 0.3% had negligible effect on growth and toxin production. At 3% glucose both bacterial growth and toxin production were suppressed. The authors hypothesized that conditions found to enhance toxin production in vitro may be present in the vagina during menses including pH, availability of complex nutrients and temperature.

Melconian et al. (1983) reported that 50 ug/ml of TSST-1 was produced in bio-trypticase medium supplemented with 1% yeast extract. Toxin concentration was determined by a microslide immunodiffusion.

Mills and coworkers (1985) found that tampon influenced production of TSST-1 in vitro was controlled by magnesium ion (Mg^{++}). Different types of tampons were added to culture medium to assess their influence on TSST-1 production. It was found that tampons composed of cotton, carboxymethylcellulose (CMC) and viscose rayon caused minimal or no change in the amount of toxin produced in comparison to controls without added fiber. In contrast, fibers from the Rely tampon composed of polyester foam (PEF) and CMC dramatically increased both growth and toxin production. The ability to enhance toxin production resided in the PEF portion of the tampon. Tampons that were made of polyacrylate rayon (PAR) inhibited growth. The growth of cultures in PAR fiber extracted BHI could be

restored by addition of Mg^{++}. When BHI was extracted with Rely fibers tenfold more Mg^{++} had to be added to restore TSST-1 production to control levels in comparison to PAR extracted BHI. Maximal toxin production was observed when 1-5 ppm was added to the PAR extracted medium. Higher levels of magnesium reduced toxin production while growth was maintained. The authors postulated that the relationship of tampons to TSS may be a result of the chelating ability of these fibers which in turn reduces the levels of magnesium in the vagina to the extent that toxin production is maximized.

Morita et al. (1979) found a similar relationship of Mg^{++} concentration to enterotoxin B production where maximum production of the toxin was reached at 5 ppm and decreased at higher levels.

Schlievert (1985) also investigated the interaction of tampons and Mg^{++} concentration on TSST-1 production and confirmed that pretreatment of medium with PAR resulted in inhibition of growth and TSST-1 production which could be reversed by the addition of 5 ppm Mg^{++}. In contrast to the report of Mills et al. (1985) higher amounts of magnesium were not found to inhibit toxin production. Rely tampons demonstrated only minimal Mg^{++} binding ability and did not inhibit growth of S. aureus or TSST-1 production.

A later study by Mills et al. (1986) examined regulation of TSST-1 and total exocellular protein production in a

chemically defined medium. Consistent with previous observations (Mills et al.,1985), low levels of magnesium allowed growth of the organism and increased the production of TSST-1 and total exoproteins. Higher levels of magnesium increased proliferation and decreased secretion of exocellular proteins. Increased production of exocellular proteins may therefore be an adaptive response to environments with insufficient essential nutrients as a means to increase utilization.

Kass et al. (1987) reported that TSST-1 was not produced in the absence of oxygen. Using a chemically defined medium it was found that at levels of O_2 from 5-20% maximal toxin levels were reached when the Mg^{++} concentration was 0.5 ug/ml. CO_2 was also found to enhance toxin production in comparison to those cultures without.

In a recent study by Todd et al. (1987) the conditions found in S. aureus infected sequestered sites in TSS patients were found to be nearly physiological. The concentrations of calcium and magnesium in these samples were found to be physiological to hypophysiological. An in vitro system was used to examine the influence of these conditions individually and collectively on both growth and the production of TSST-1. Optimal conditions included 6% CO_2, pH 7.0, aerobic and high levels of protein. Changing any of these conditions decreased production of TSST-1. Production of TSST-1 had a positive correlation to growth

of the organism. In media with added protein, reduction of the divalent cation concentration had less effect and maximum toxin production was observed at magnesium concentrations that were greater than physiological. These investigators stated that the pathogenesis of TSS appears to be multifactorial and requires exposure to a toxin producing organism, lack of established immunity and optimal conditions for growth and toxin production.

Tampons and toxic shock. Broome et al (1982) examined the interactions of tampons on the growth of S. aureus. Addition of Rely and Tampax super tampon fibers to TSB did not enhance growth in comparison to control cultures without added fiber. Tampax super plus tampons inhibited the growth of the test organism within 4 hr. Addition of blood to the medium alleviated this effect and the cultures in the presence of the super plus tampons grew as well as the controls. Tampons were examined for possible intrinsic contamination as it had been postulated that tampons may act as fomites and introduce the causative organism into the vagina. Although all the tampons tested were found to have S. epidermidis and Bacillus species present no S. aureus was isolated. The persistence of S. aureus inoculated onto different brands of tampons was examined and the bacteria were found to survive the longest on Rely tampons and the shortest on OB super plus. The authors

concluded that tampons do not have a significant effect on the growth of S. aureus in vitro and that enhancement of growth may not be a factor in the development of the syndrome. Intrinsic contamination as a factor could not be ruled out because with low frequency contamination at the rate of 7 tampons per 1000 would be consistent with the frequency of the disease occurring at that time in 6 out of 100,000 menstruating women each year. The intrinsic contamination theory is supported by the ability of the Rely tampon maintain viability of toxin producing S. aureus for longer than the other brands.

Ingham et al. (1985) investigated the effects of different tampon materials on the physiology of a TSS associated strain of S. aureus (FRI 1187) using an in vitro system. The culture medium used for the study was BHI supplemented with sterile salts. Fibers were added to the medium at 25 g/500 ml. Five out of the seven fibers tested (5 types of rayons, cotton, CMC) reduced the growth rate of the test organism. Two of the rayons did not affect the growth. Levels of phosphatase, lipase, proteinase, hyaluronic lyase were determine in filtrates from fiber grown cultures. Cotton and CMC reduced the levels of all enzymes, while other fibers had variable effects. All of the fibers decreases lethality of the culture filtrates in comparison to the controls when injected into BALB C mice. Although the effect of these fibers on TSST-1 production

was not determined, it was theorized that since each fiber had distinct effects on the level of specific enzyme production and growth of the organism, that the type of fiber used in tampons could have an impact on the development of TSS.

Complementing the previous work by Ingham et al.(1985), Holland et al. (1985) continued studies on rayon and CMC which were described earlier as examples of fibers with minimal and maximal effect, respectively, on the physiology of S. aureus in vitro. This study confirmed the effects seen in the first study using a different procedure. The fibers were used to pretreat the medium and were then removed and incubation continued. Rayon treated cultures had minimal effect on exoenzyme or toxin production while pretreatment with CMC increased exoprotein production. It was thought that these results were due to removal of nutrient(s) from the medium by the superabsorbent CMC.

In a report by Schlievert and colleagues (1985) the effect of different tampons on the production of TSST-1 was examined. Under conditions known to produce high levels of toxin including culture in dialyzable beef heart medium with or with out blood, aeration, and a pH of 7-8, the influence of tampons on toxin production was examined. Lower absorbency tampons were found to decrease the amount of toxin produced while having little effect on growth. High absorbency tampons were found to have variable effects

including no effect on growth, reduction of toxin production (OB super plus), inhibition of both growth and toxin production (Kotex security super, Rely super and Playtex super) and inhibition of growth with more toxin produced per cell (Tampax super plus). Tampons added to the medium did not, in any case, stimulate higher levels of toxin production than controls without added fiber.

Tierno and Hanna (1985) investigated the levels of TSST-1 produced in the presence of various tampons and a contraceptive sponge using BHI supplemented with 1% yeast extract or 20% blood. The greatest amount of TSST-1 was produced in the presence of the Rely tampon as determined by a radial immunodiffusion assay. All of the tampon products including several absorbencies from six major brands increased the amount of TSST-1 produced over that of the controls. The levels of TSST-1 were greater in the presence of blood. The Today contraceptive sponge inhibited growth in BHI but the presence of blood overcame the inhibition and TSST-1 was detected.

In a controversial study, Tierno and coworkers (1983) hydrolyzed the CMC component of Rely with cellulase enzymes including beta-glucosidase and cellulase and found that the degradation products supported the growth of a TSS associated strain of S. aureus. It was observed that CMC was hydrolyzed by beta-glucosidase to yield glucose. According to the authors, there are many organisms in

addition to human tissue which produce this enzyme. The effect of the enzyme in the vagina in the presence of Rely CMC would be to provide an exogenous nutrient source and thus enhance growth of pathogenic organisms.

Later, Bonventre (1983) commented that there is already sufficient glucose present in the vagina to support the growth of S. aureus without further degradation of a tampon. Kirkland and Widder (1983) also responded to the paper of Tierno and demonstrated the presence of cellulases as contaminants in the beta-glucosidase preparation used in the study of Tierno and Hanna. Beta-glucosidase does not break down CMC by itself and no cellulases were known to be produced by vaginal microflora so the significance of this finding was questioned. Aerobic and facultative anaerobes isolated from the vaginal tract were screened for cellulase activity by Garland and coworkers (1987). Of 288 isolates, none were found to be cellulolytic. These authors concluded that it was unlikely that cellulolytic organisms have an impact on cellulosic catamenial products in the vagina.

Lee and coworkers (1987) examined the effect of tampons on production of TSST-1 in vitro using a syringe as a culture vessel. Three ml of medium was added per gram of tampon fiber. It was believed by the authors that the syringe method was closer to the physical conditions found in vivo than other culture methods. The authors concluded

that CO_2 is an important factor for TSST-1 production as two times more TSST-1 was produced with its addition. The addition of blood also promoted both increase in growth and TSST-1 production. These authors found that PAR which has been implicated in TSS did not stimulate as much TSST-1 production as some of the cotton-rayon tampons. Rely tampons were found to induce the greatest production of TSST-1 of all the products tested and the toxin stimulatory portion was found associated with PEF not CMC.

Vaginal microflora. Although the literature is replete with studies on vaginal microflora, there are few reports on qualitation and quantitation of bacteria during menses. A report by Onderdonk et al. (1986) examined the microflora of menstruating women using menstrual pads or cotton tampons and found that the total bacterial counts decreased during menses. Cotton tampons had minimal effect on the microflora. The anaerobic counts remained relatively stable throughout the cycle regardless of the type of catamenial product used. The finding that the counts on the vaginal mucosa were higher than detected on the tampon led the authors to suggest that the tampon did not serve as a focus for growth. During menses the majority of organisms were gram positive in contrast to intermenstrual samples which were predominately Lactobacillus species.

In a survey on vaginal colonization by Escherichia coli

(Chow et al.,1986) the vaginal carriage rate of this organism increased during and immediately after menses. Interestingly the rate of vaginal carriage of E. coli was higher among women who carried TSST-1 producing S. aureus than non toxigenic strains or no staph at all. The total counts remained high throughout the menstrual cycle.

Vaginal ulcerations. The relationship between tampon use and TSS has prompted studies on this association as a contributing factor to the development of the disease. Friedrich (1981) theorized that retrograde absorption of toxin through the endometrium is unlikely while absorption across the intact vaginal epithelium is possible. It is also possible that damaged membrane is necessary for the toxin to traverse into the circulatory system. It was reported by the CDC (1980) that superabsorbent tampons, more frequently associated with TSS, were also more likely to cause microulcerations. Another causal relationship for tampons and TSS is that a majority of the TSS patients were found to be colonized by S. aureus intralabially. In these women, introduction of the tampon may concomitantly carry staphylococci into the vagina.

In a study by Berkely et al. (1985) tampon users were found to have significantly more vaginal drying and layering than external pad users. These effects were considered to be a physiological response of the vagina to

menstruation in the presence of a tampon and as such are relatively benign. The occurrence of ulceration was found to be an uncommon event occurring in 4 out of 60 subjects who used superabsorbent (polyacrylate rayon) tampons in comparison to rayon or cotton tampon users (1/180). This was consistent with an earlier study by Friedrich and Siegesmund (1980) who also found an increased occurrence of ulcerations in subjects using superabsorbent tampons.

In view of the chelating ability of certain tampon fibers (Mills et al.,1985) it is interesting that in an investigation by Schuchner and coworkers (1974) examining the effect of EDTA impregnated tampons on the vaginal epithelium the effect of the treated tampons appears similar to the effect of certain superabsorbent tampons. These authors noted a marked opening of the desmosomes and increase in the size of the intracellular spaces in addition to splitting and separation of the cells.

C H A P T E R II

MATERIALS AND METHODS

Organisms. Strains of Staphylococcus aureus used in this study were obtained as follows: non-toxic shock syndrome (TSS) associated S. aureus 6538-p from the American Type Culture Collection (Rockville, Maryland), isolates from tss patients: S. aureus 587 from Dr. Jean Lee (Harvard Medical School, Boston MA); S. aureus MN-8 from Dr. Patrick Schlievert (University of Minnesota Medical School, Minneapolis, Minn.); S. aureus strains 189 and H219, a TSS associated organism and a non-TSS associated vaginal isolate, respectively, from Dr. Robert Walker (University of Massachusetts, Amherst, MA).

Strains in frequent use were maintained on tryptic soy agar (TSA, tryptic soy broth with 1.5% agar, Difco, Detroit MI) slants and transferred to fresh slants every 2-3 months. For long term storage, an overnight culture in tryptic soy broth (TSB, Difco) was divided into 0.5 ml aliquots, flash frozen in an acetone-dry ice bath and stored at -70°C.

Enumeration of bacterial cultures was accomplished by serial dilutions into 0.1% peptone (Difco) water or TSB and subsequent plating onto TSA plates using the smear plate technique. All plates were incubated for 24 hr at 35°C

before counting colony forming units (cfu).

In early experiments on purification of toxic shock syndrome toxin-1 (TSST-1), the toxin producing strains were grown on brain heart infusion (BHI, Difco) dialysate. This medium was prepared by dissolving 148 g BHI in 400 ml H_2O and putting this solution into dialysis tubing with a molecular weight retention limit of 3500 kilo Daltons (kDa). The filled dialysis tubes were put into 3600 ml H_2O and steamed for 4 hr. The resulting dialysate was divided into 600 ml aliquots and autoclaved in 2.8 l Fernbach flasks. The purpose of this procedure was to control the molecular weight of the proteins supplied by the growth medium and minimize interference from the medium proteins during the purification procedure. When it was later found that undialyzed BHI contained minimal amounts of proteins with molecular weights greater than 10 kDa, preparation of the medium in this fashion was discontinued. The bacteria were then propagated in undialyzed BHI broth.

For experiments investigating the influence of different tampon fibers on toxin production, fiber was handled with forceps to minimize contamination and weighed out into sterile petri dishes. The fiber was transferred to a sterile plugged 125 erlenmeyer flask or a screw top tube and stored at room temperature until needed. Sterile BHI was added to the preweighed fiber immediately before inoculation using 10% fiber (w:v).

Preparation of fibers. Samples used in this study consisted both of fibers before processing into tampons and fiber pieces separated from whole tampons. Fiber samples were supplied by Tambrands Inc (Palmer MA) as were some of the packaged tampons. Others were purchased commercially. Samples of Rely tampons were obtained from Dr. Robert Walker and from Mary Kendrick (Channing Laboratory, Harvard Medical School, Boston MA).

The Rely tampon was composed of a polyester overwrap, polyester foam (PEF), carboxymethylcellulose (CMC) chips and a polyester withdrawal cord. The CMC chips tended to break up and disperse throughout the other components. In order to evaluate the magnesium binding ability of PEF and CMC separately it was necessary to wash the PEF exhaustively in H_2O to completely remove the CMC. The PEF was repeatedly squeezed out in fresh distilled water until no viscosity could be detected in the rinse water. For the purpose of removing the shorter soluble dextrans from the colloidal CMC an exhaustive washing procedure was carried out on this component also. The CMC chips were rehydrated in H_2O, shaken thoroughly and centrifuged. The supernatant was discarded and more H_2O added. This process was repeated until the supernatant had no discernible viscosity. The washed CMC was lyophilized so that the weight per ml would be quantitated.

In animal studies where Rely PEF was implanted intravaginally, native (unwashed) PEF was used. The cotton for these experiments was cut from Original regular tampons (Tambrands Inc). Both types of fiber pieces were covered in the Rely polyester overwrap and tied with a piece of Rely withdrawal cord. The implants were, therefore, exactly the same except for the PEF and cotton.

Animals. The guinea pigs used in this study were Hartley strain 13 which are inbred and tested psittacosis free. The animals were housed in groups of 1-4 depending on the number of replicates in a specific experiment. There was free access to pelleted guinea pig rations and water at all times.

Guinea pigs were bled by cardiac puncture under ether anaesthesia. The blood samples were collected into Corvac blood collection tubes and allowed to clot for about 30 min at room temperature. Tubes were centrifuged for 15 min at 3000 rpm to separate the serum from the clot. Serum samples were stored frozen at -20^{o}C until assayed.

Tears were collected by tilting the animal's head downward allowing the tears to collect in the corner of the eyes. The secretions were collected with a 20 ul capillary tube and then expelled into a microcentrifuge tube and capped. These samples were stored at -70^{o}C until assayed.

Intravaginal inoculation of the guinea pigs was performed

under anaesthesia. A sterile plastic microcentrifuge tube about 3 mm in diameter was used to dilate the vaginal opening for introduction of the fiber pieces. A micropipet was used to deliver 20 ul of inoculum into the vagina. The fiber samples were recovered 2 days later with forceps.

A New Zealand white rabbit and a goat were both used for produce specific anti-TSST-1 antibodies. The rabbit was immunized with antigen in Freund's complete adjuvant subcutaneously in 12 sites on its back. Three weeks later a second vaccination was given using Freund's incomplete adjuvant. Blood was collected through an ear vein and serum separated in a Corvac blood tube as previously described.

The goat was immunized with antigen suspended in Freund's complete adjuvant, subcutaneously and intramuscularly, in about 20 sites in the hindquarters. A second inoculation was given 2 weeks later also using complete adjuvant. Blood was collected from the jugular vein into a 500 ml evacuated bottle. Antibody to TSST-1 was detected by double immunodiffusion assay and radioimmunoassay (RIA) 4 weeks post inoculum. Three additional collections of serum were made 4 days apart.

Radioiodination. Preparations of TSST-1 were iodinated with equal success by either of 2 methods. Iodination with ^{125}I-Bolton-Hunter reagent (NEN, North Billerica, MA) was performed by reacting 10 ug of TSST-1 preparation in 100 ul

0.05 M sodium phosphate buffer pH 8.0 with 1 mCi ^{125}I reagent after the benzene solvent was evaporated. The reaction vial was held in an ice bath for 1 hr and then terminated by adding 0.2 ml of 0.2 M glycine in 0.05 M sodium phosphate buffer pH 8.0. The labelled TSST-1 was separated from unbound reagent by gel filtration through a Sephadex G-25 column (0.5 cm x 18 cm) equilibrated with 0.05M sodium phosphate pH 7.5 with 0.85% NaCl. One ml fractions were collected and the labelled protein eluted in the void volume while the unbound label was held up in the gel. A 10 ul aliquot of each fraction was counted on a Beckmann gamma counter (model 4000). Trichloroacetic acid (TCA) precipitation was used to determine protein bound radioactivity. Addition of 1.0 ml of 1% bovine serum albumin (BSA) in 0.05 M sodium phosphate buffer as a carrier and an equal volume of 20 % TCA in H_2O resulted in a precipitate. The precipitate was centrifuged, washed twice with 10 % TCA and counted. The percent TCA precipitable counts obtained with the Bolton-Hunter procedure ranged from 50-75% with 80-90 % of the precipitable counts immunoreactive as determined by RIA. The labelled fractions were stabilized with 10 mg BSA per ml and stored at $4^{o}C$.

The second iodination method utilized the enzymatic lactoperoxidase-glucose oxidase procedure with Enzymobeads (Biorad, Richmond, CA.). TSST-1 preparations to be

iodinated using Enzymobeads were dialyzed against 0.05 M sodium phosphate buffer pH 7.5 with 0.85 % NaCl overnight in the cold. The protein preparation was adjusted in concentration to 10 ug/100 ul. Fifty ul of rehydrated Enzymobeads was added to 100 ul of the buffered protein along with 1 mCi $Na^{125}I$. The reaction was initiated by adding 25 ul of 1 % B-D-glucose. The mixture was stirred on a magnetic stirring plate at room temperature for 2.5 hr. The labelled TSST-1 was separated from unbound $Na^{125}I$ by gel filtration on Sephadex G-25 and tested for percent TCA precipitable counts as previously described. The percent of immunoreactive TCA precipitable counts by this method was comparable to the results obtained with the Bolton-Hunter procedure.

Radioimmunoassays. The assay mixture for titering sera by radioimmunoassay (RIA), consisted of the following; 20 ul of serum diluted into 1% BSA in phosphate buffered saline (PBS, 0.05 M NaH_2PO_4 adjusted to pH7.5 with dilute KOH, 0.85% NaCl) and 100 ul ^{125}I-TSST-1 (10,000 cpm). This was mixed on a Vortex mixer and incubated on a shaking platform for 1 hr at room temperature. Fifty ul of fixed S. aureus cowan 1 strain (ATCC # 12598) consisting of 10 % packed cell volume were added to each assay tube as an immunoprecipitate. The assays were incubated for an additional 30 min and centrifuged at room temperature for

15 min at 3000 rpm. The supernatant was aspirated and the pellet washed twice using 2 ml of PBS, centrifuging after each wash and counted. The titer of an immune serum was the last dilution having a average count 2 SD greater than counts from control assays with prebleed or normal sera.

Competitive inhibition assays to determine the relative amount of TSST-1 present were performed using the same protocol except that the order of addition was changed. In these assays, 50 ul of sample containing unknown amounts of TSST-1 was mixed with an equal volume of iodinated toxin. Twenty ul of rabbit anti-TSST-1 antibody, at a working dilution of 1/160, was added, Vortexed and processed as previously described.

Preparation of fixed cowan 1 cells. The procedure for preparation of fixed S. aureus cowan 1 cells as an immunoprecipitant was based on that of Miller et al. (1978). Ten ml of a 24 hr culture of S. aureus cowan 1 strain (ATCC #12598) grown in TSB, was used to inoculate each of two 2.8 l Fernbach flasks containing 1000 ml of TSB. The inoculated culture vessels were incubated at 35°C for 24 hr on a rotating shaker. The cells were harvested by centrifugation at 8000 rpm and the pellets washed twice with PBS containing 0.05 % sodium azide (PBS/azide). The pellets were resuspended and pooled in a total of 100 ml PBS with 2 % formaldehyde. The cell suspension was stirred

overnight at 4°C. The fixed cells were washed twice with PBS/azide, heated at 80°C for 5 min while stirring and plunged into an ice bath. The cells were washed two additional times with PBS/azide. After the final wash and centrifugation, the cells were suspended in sufficient PBS/azide so that the packed cell volume was 10 % of the total. Aliquots of the fixed cell suspension were flash frozen in a dry ice/acetone bath and stored at -20°C. Before use the cells were thawed rapidly in a 30°C waterbath, washed once and resuspended in PBS/azide.

Immunodiffusion assays. Double immunodiffusion was performed in 0.9% Gelrite or 1 % agarose in PBS in 47 mm petri plates or with the gel poured on Mylar sheets. Wells were cut and plugs aspirated immediately before use. Immunoprecipitation lines were allowed to form for a minimum of 48 hr at 4°C. The gels were washed for another 48 hr using at least 5 changes of PBS and stained for 30 min in 0.025 % coomassie brilliant blue R 250 in methanol:acetic acid:water, 50:45:5 by volume and destained in methanol:acetic acid:water, 87:8:5 by volume.

Enzyme-linked immunosorbant assay for TSST-1. The competitive ELISA for quantitation of TSST-1 in experiments on the effect of different tampon fibers on toxin production was conducted according to the procedure of

Parsonnet et al.(1985). TSST-1 was conjugated to alkaline phosphatase in PBS using 20 ul of 4 % glutaraldehyde and stirring for 2 hr. The solution was then diluted to 2 ml and dialyzed against PBS. The conjugate was separated from unconjugated protein by gel filtration on a Ultragel AcA 44 column (2.6 x 40 cm) and fractions assayed for both enzyme and immunoreactivity by incubating an aliquot in a microtiter plate well coated with rabbit anti-TSST-1 antiserum, washing and adding substrate. Fractions positive in this assay were pooled, adjusted to 1 % BSA and 0.1 % in sodium azide and stored at 4^{o}C.

Microtiter plates (Immulon-2, Dynatech, Alexandria, VA) were coated with 200 ul of TSST-1 antibody diluted in 0.05 M carbonate buffer pH 9.6 for 4 hr at 37^{o}C, and washed three times with PBS/0.02 % tween 20. The sample to be tested was diluted, and 100 ul added to duplicate wells. The plates were incubated overnight at room temperature in a humidity chamber. The plates were washed three times with PBS/tween 20 and 200 ul of para-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine pH 9.8 added to each well. The plate was incubated at 37^{o}C for 1 hr or until the control well containing conjugate with no inhibitor reached an absorbance (405 nm) of 1.0. At this point the entire plate was read in an ELISA plate reader (model EL 307, Titertek). The amount of TSST-1 per sample was calculated by plotting the absorbance against a standard curve of

absorbance versus the log of known concentrations of purified TSST-1 run in each assay. The antilog of the concentration times the dilution factor was equal to the ug of TSST-1 per ml.

Polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli(1970). For separation of low molecular weight proteins a 10 % acrylamide separating gel pH 8.8 and a 6 % acrylamide stacking gel, pH 6.8 was used. The ratio of acrylamide to bis-acrylamide was 30:0.8. Gels were prepared from stock solutions of:

#1. 30 g acrylamide, 0.8 g bis-acrylamide

#2. 1.5 M tris-HCl pH 8.8

#3. 10 % SDS

#4. 0.5 M tris-HCl pH 6.8

A 10 % separating gel was made with 8.3 ml sol. #1, 6.3 ml sol. #2, 250 ul sol. #3 and 10.2 ml H_2O. The solution was degassed under vacuum and 65 ul of freshly prepared 10% ammonium persulfate and 25 ul of N,N,N',N'-tetramethylene diamine (TEMED) added immediately before pouring the gel. The stacking gel was made with 2.0 ml sol. #1, 0.1 ml sol. #3, 2.5 ml sol. #4, 10 ul TEMED and 100 ul of 10 % ammonium persulfate. The running buffer was composed of 0.05 M tris-0.38 M glycine, 0.1 % SDS pH 8.8.

Samples were prepared for electrophoresis by adding an equal volume of sample buffer (2.5 ml of 0.5 M tris- HCl, pH 6.8, 2.0 ml 10 % SDS, 2.0 ml glycerol, 0.1 ml 0.1 % bromophenol blue and 3.2 ml H_20) and 15 % beta-mercaptoethanol and boiling for 5 min. Gels were run at 4°C and electrophoresed for 40 min at 60 v and then at 120 v (constant voltage) until the tracking dye reached the bottom of the gel.

Non-denaturing gels were made with the same solutions except that SDS and B-mercaptoethanol were omitted and samples were not boiled before electrophoresis.

Gels were stained using the quick stain procedure of Calabrese et al (1980). Staining was carried out in 0.2 % coomassie blue in methanol/acetic acid/water (5:1:5 by volume) in a 55°C water bath for 60 min and destained at the same temperature in several changes of methanol/acetic acid/water (7:2:26 by volume). When separation on nondenaturing gels was used as a final purification step for TSST-1, prior to using the protein for animal immunization, the sides of the gels were cut, stained for about 10 min, and then destained for about 10 min. The stained ends were realigned with the unstained portion of the gel and the desired band cut out. This procedure allowed the protein bands to be rapidly discriminated and minimized diffusion in the unstained portion of the gel.

Low molecular weight protein standards (Sigma, St.

Louis,MO) consisted of BSA (MW 66 kDa), egg albumin (MW 45 kDa), glyceraldehyde-3-phosphatase dehydrogenase (MW 36 kDa), carbonic anhydrase (MW 29 kDa), trypsinogen (MW 24 kDa), soybean trypsin inhibitor (MW 24 kDa) and alpha-lactalbumin (MW 14.2 kDa).

Samples to be silver stained were prepared as previously described and separated on precast 10-15% continuous gradient SDS gels using a Phastgel system (Pharmacia, Piscataway, NJ) with a discontinuous tris-tricine acetate buffer system (Laemmli,1970). The gels were stacked for 10 Vhr and separated for 50 Vhr. Silver staining was performed according to the method of Heukeshoven and Dernick (1985) modified for automation by the manufacturer (Pharmacia). Isoelectric focusing to determine the pI of TSST-1 was also performed on the Phastgel system using precast gels with a pH gradient of 3 to 9. The resolved gels were silver stained. The pI calibration standards (Biorad) that were used contained alpha-chymotrypsin (pI 8.8), whale myoglobin (pI 8.05), equine myoglobin (pI 7.0), human carbonic anhydrase (pI 6.5), bovine carbonic anhydrase (pI 6.0), beta- lactoglobulin B (pI 5.1) and phycocyanin (pI 4.65). The isoelectric point of TSST-1 was calculated against a standard curve where the pI of the standards was plotted versus distance in mm moved from the cathode.

Immunoblots. Proteins resolved by PAGE were transferred onto 0.45 micron pore size nitrocellulose membranes (Schleicher and Schuell Inc, Keene NH) using the procedure of Towbin et al (1979). The polyacrylamide slab was washed in 2 changes of transfer buffer (18.17 g Tris-HCl; 86.5 g glycine; H_2O to 4800ml; 1200 ml methanol) for 30 min. The gel was placed on a piece of filter paper on top of a scotch-brite pad, covered with a sheet of nitrocellulose membrane, (soaked in transfer buffer) a second sheet of filter paper and another scotch-brite pad. The sandwich was locked into place between plastic sleeves and placed in a electrophoresis chamber filled with cold transfer buffer. The proteins were transferred onto the membrane for 2.5 hr at 55 v (constant voltage).

On completion of the transfer, the area of the membrane containing the standards and a portion of the sample were cut off, stained with Amido black (1 g Amido black in H_2O:ethanol:acetic acid, 50:40:10 by volume) and destained in H_2O:ethanol:acetic acid, 50:40:10). The remainder of the membrane was blocked with 3 % BSA in 20 mM Tris pH 7.5 containing 0.85 % NaCl (TBS) for 1 hr at 30^{o}C on a rocking platform shaker. The membrane was washed 3 times in TBS containing 0.1 % BSA and 0.05 % tween 20 (TBS wash). To examine the immunoreactivity of specific sera, each sample was diluted in TBS with 1 % BSA and 0.05 % tween 20 (Ab diluent). The diluted sera were incubated with a strip of

the membrane for 2 hr at 30°C or 18 hr at 4°C. The membranes strips were washed 3 times in TBS wash and incubated for 1 hr at room temperature with a second antibody conjugated to horseradish peroxidase and diluted 1/1000 in Ab diluent. The membrane strips were washed 3 times in TBS wash and developed with a mixture of H_2O_2 and 4-chloro-naphthol (comercially prepared solutions, 1:1 by volume, Kierkegard and Perry, Gaithersberg, MD). Color development was stopped by rinsing the membranes in H_2O after they were sufficiently stained (5-15 min).

Immunoglobulin purification. Goat and rabbit sera were complement inactivated by heating at 56°C for 20 min. Lipids were removed by centrifugation for 20 min (12000 rpm) at 4°C and decanting the serum through glass wool to remove insoluble lipids. The IgG was precipitated from the clarified serum by slowly adding dry ammonium sulfate while stirring at room temperature to 55 % saturation. The solution was stirred for 30 min to allow the precipitate to fully form. The precipitate was separated by centrifugation for 20 min at 12,000 x g. The pellet was dissolved in a minimal amount of BSB, pH 8.0, and dialyzed against the same buffer overnight in the cold. The protein concentration was determined by the method of Bradford (1976) using prepared reagent (Biorad). Purified bovine IgG was used to generate the standard curve. The crude

IgG was further purified by gel filtration through a Sephadex 6B-Cl column (1.5 x 85 cm) equilibrated with BSB. The sample was applied in a volume about 1/100 of the total column volume. One ml fractions were collected at a rate of 1 drop/8 seconds. Fractions were monitored for absorbance at 280 nm and tubes containing protein were assayed by RIA or immunodiffusion to confirm immunoreactivity against TSST-1.

Affinity chromatography. Purified goat anti-TSST-1 IgG in 0.1 M Mops pH 7.5, was coupled to Affigel-10 affinity support (BioRad) using 15 mg protein per ml gel volume. Prior to coupling, the Affigel-10 was rinsed with 2 bed volumes of cold 2-propanol followed by 3 bed volumes of cold H_2O. The gel was added to the solution of goat IgG and rotated end over end for 18 hr at 4°C. To block any active esters that might be remaining, 0.4 ml 1 M ethanolamine-HCl pH 8.0 was added and the gel suspension rotated for another hr. The gel was poured into a column and rinsed with 50 ml of 0.1 M Mops pH 7.5. followed by 10 ml of elution buffer (0.1 M glycine-HCl pH 2.5). The column was washed with 10 mM tris-HCl with 0.15 M NaCl, pH 7.5 (binding buffer) until the absorbance of the eluant at 280 nm was zero.

For affinity purification of TSST-1, the sample was concentrated by ammonium sulfate precipitation (0-90% cut)

and dialyzed overnight in 3500 kDa MW cutoff dialysis tubing against binding buffer. The sample containing 10-15 mg total protein was run through the column at a rate of 1 drop/15 seconds. Upon completion of sample application, the column was rinsed with binding buffer until the absorbance (280 nm) was close to zero. The TSST-1 binding to the column was eluted with 5 ml elution buffer. Change in the optical density of the fractions was monitored and the peak fractions were pooled and dialyzed against binding buffer. The degree of purification was assessed by SDS-PAGE and coomassie blue staining.

Measurement of magnesium ion concentration. In experiments where magnesium ion concentration was determined, two procedures; the colorimetric method of Smith (1954), and atomic absorption spectrophotometry, were used. These procedures were found to give comparable results although differing in sensitivity. The colorimetric procedure assay mixture included 1.0 ml of sample diluted in deionized water, 1.5 ml ammonium chloride-ammonium hydroxide buffer pH 10.2 (6.75 g NH_4Cl, 57 ml concentrated NH_4OH made to 1 l in H_2O), 0.2 ml 0.44 % Eriochrome black T and 7.3 ml of H_2O. The assay mixture was vortexed and read at an absorbance of 520 nm. A standard curve was prepared using a stock solution of $MgCl_2$ with a range of 0-10 ug Mg^{++} per ml. Samples analyzed by atomic absorption

spectrophotometry were diluted in deionized H_2O so that the Mg^{++} ion concentrations were 0.1-1.0 ug per ml and then measured in a Perkin-Elmer atomic absorption spectrophotometer (model 303, Perkin-Elmer, Norwalk,CN). Standard solutions containing 0.1-1.0 ug/ml Mg^{++} in the form of $MgCl_2$, were analyzed and used to plot a standard curve.

C H A P T E R III

RESULTS

Purification and Characterization of TSST-1

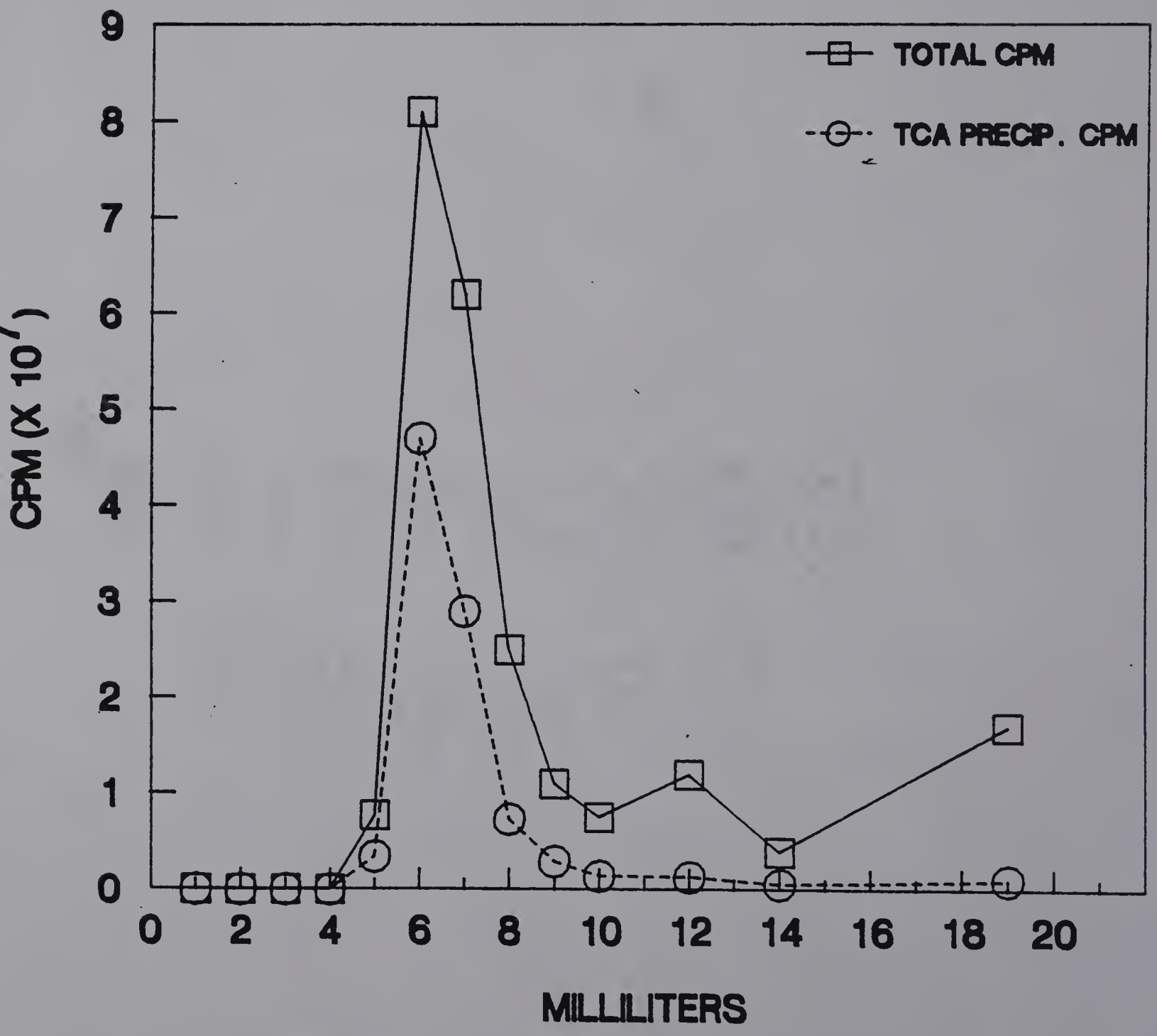
Identification of TSST-1. Partially purified TSST-1 received for use in immunodiffusion assays was found to be insufficiently pure to be used as a standard for purification of additional toxin. Since the crude preparation resolved to at least five bands with SDS-PAGE it was therefore necessary to identify which of the proteins corresponded to TSST-1.

In order to confirm which band represented TSST-1, the partially purified preparation was iodinated with ^{125}I Bolton-Hunter reagent. The labelled protein mixture was separated from unreacted label by passage through a Sephadex G-25 column. The proportion of label that was bound to protein in each fraction was determined by precipitation with trichloroacetic acid (TCA). Total and TCA precipitable counts per minute (cpm) for each fraction are presented in Figure 1.

Fraction 6 which contained the most labelled protein was reacted with rabbit anti-TSST-1 antiserum. The rabbit antiserum received as a standard preparation (FS-12, MS Bergdoll) was monospecific for TSST-1. The reaction mixture contained about 1 million cpm from fraction 6 and

Figure 1. Elution profile of ^{125}I labeled TSST-1 from a sephadex G-25 column. Ten ug of a TSST-1 preparation was labeled with 1 mCi Bolton-Hunter reagent. The reaction mixture was chromatographed through a 1 X 10 CM sephadex G-25 column. Fractions eluting from the column were monitored for total counts (—⊟—), and TCA precipitable counts (-⊖-).

Figure 1


TOTAL CPM
TCA PRECIP. CPM
CPM (X 10^7)
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
20
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0.1 ml of rabbit antiserum and was reacted with 50 ul of protein A-sepharose to precipitate out IgG bound-labelled protein. After washing the pellet in additional PBS both the pellet and supernatant fractions were reduced and solubilized in a SDS-sample buffer containing beta-mercaptoethanol. After separation by SDS-PAGE, the bands of labelled protein were visualized by autoradiography. As shown in Figure 2, the anti-TSST-1 antiserum was monospecific, absorbing only one band from the crude protein preparation (lane 1). The adsorbed protein band had a relative mobility corresponding to a molecular weight of 22 kDa. The molecular weight of this band is consistent with descriptions of TSST-1 in the literature (Bonventre et al,1983). Lane 2 consists of the supernatant after absorption of the IgG-labelled protein complex with protein A. The band which was absorbed by the antiserum has decreased in intensity in comparison to the control lane 3 where normal rabbit serum was used instead of the rabbit anti-TSST-1 preparation.

An attempt was made to purify TSST-1 by absorption of the iodinated crude preparation with the specific antiserum and then dissociation of the resulting complex with 1.0 M propionic acid. The IgG-labelled-TSST-1 complexes were separated from the non-TSST-1 labelled proteins with protein A-sepharose. Finally TSST-1 was dissociated by the addition of propionic acid and separated from IgG by gel

Figure 2. Autoradiogram of ^{125}-I TSST-1 reacting with rabbit antiserum. TSST-1 (1 X 10^6 cpm, Fig. 1, #6) was immunoprecipitated with 100 ul rabbit antiserum and 10% protein-A suspension.
Lane 1. label bound by rabbit anti-TSST-1 serum. Lane 2. supernatant after absorbtion with specific rabbit antiserum. Lane 3. supernatant after absorption with non-immune rabbit serum.


22 kDa
1
2
3

filtration on a Sephadex G-75 column. Two peaks were delineated when fractions were monitored for ^{125}I activity on a gamma counter. The first peak shown in Figure 3 corresponds in molecular weight to the undissociated IgG-labelled TSST-1 complexes eluting at the void volume. The second peak at fraction 81, consisted of the labelled TSST-1.

Once the elution profile of TSST-1 was determined for the Sephadex G-75 column additional iodinated toxin was separated from the crude preparation using this method of chromatography. At least three peaks were obtained when the crude toxin preparation was separated on the Sephadex G-75 column (Figure 4). The second peak eluted at fraction 81 which corresponds to the same peak fraction for the labelled TSST-1 that was bound by the rabbit anti-TSST-1 serum. Fractions 75-82 were pooled and used as a tracer for tracking TSST-1 in a large scale purification.

Isolation of TSST-1. The initial isolation of TSST-1 was conducted using a modification of the protocol described by Notermans and Dufrenne (1982). Diluted culture supernatant from TSST-1 producing S. aureus strain MN-8 was adjusted to pH 5.7 and absorbed with 250 ml of preswollen CM sephadex C-25. The slurry was stirred for 2 hr at room temperature and allowed to settle before decanting the supernatant. The gel was poured into a column, rinsed and

Figure 3. Elution profile of TSST-1 from sephadex G-75. Labelled TSST-1 (10^6 cpm, Fig 1, #6) was immunoprecipitated from a crude toxin preparation with 100 ul rabbit anti-TSST-1 serum and 10% protein-A. The resulting complexes were dissociated with 1 M propionic acid and separated over a sephadex G-75 column (1.5 x 85 cm).

Figure 3

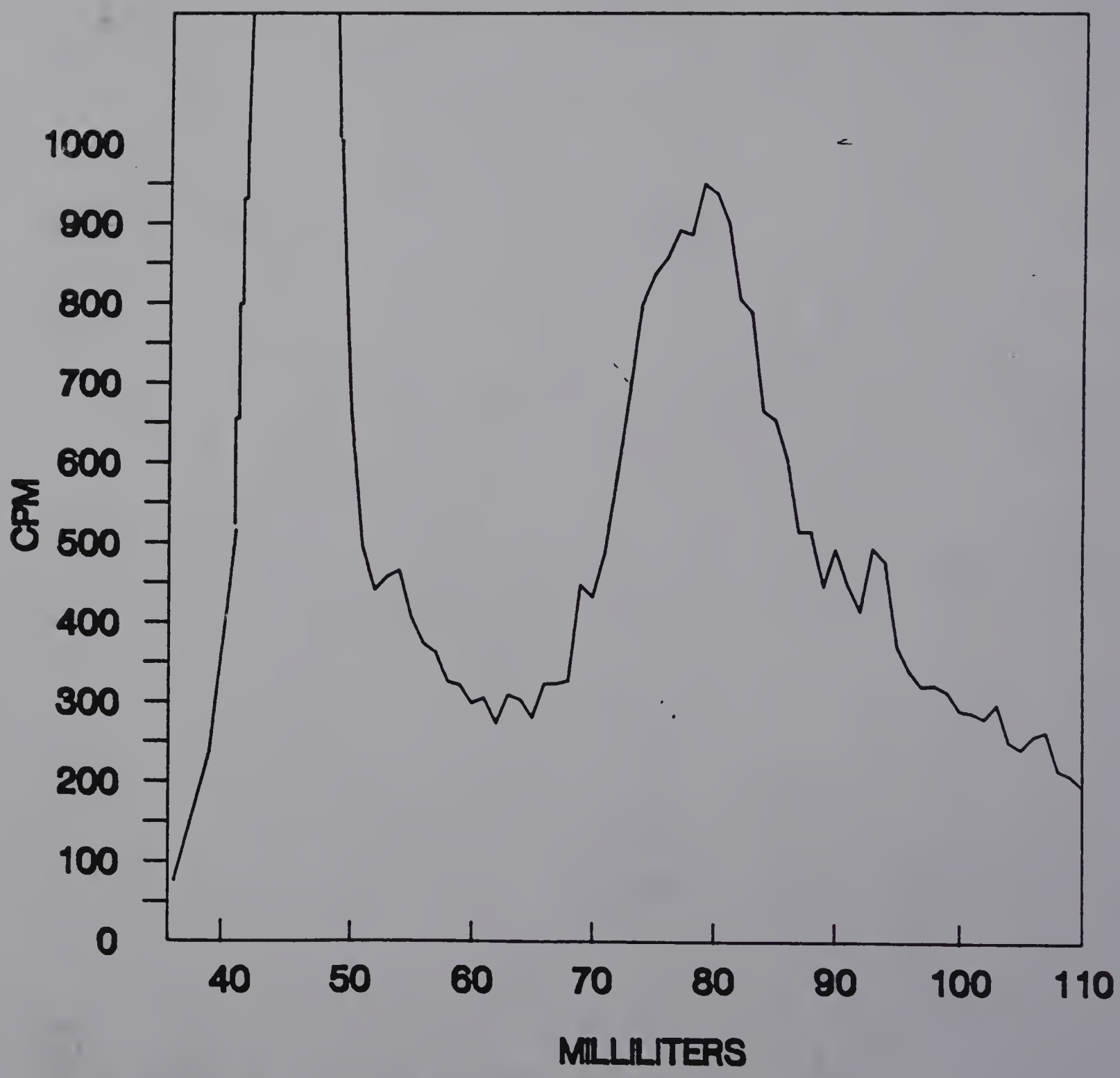
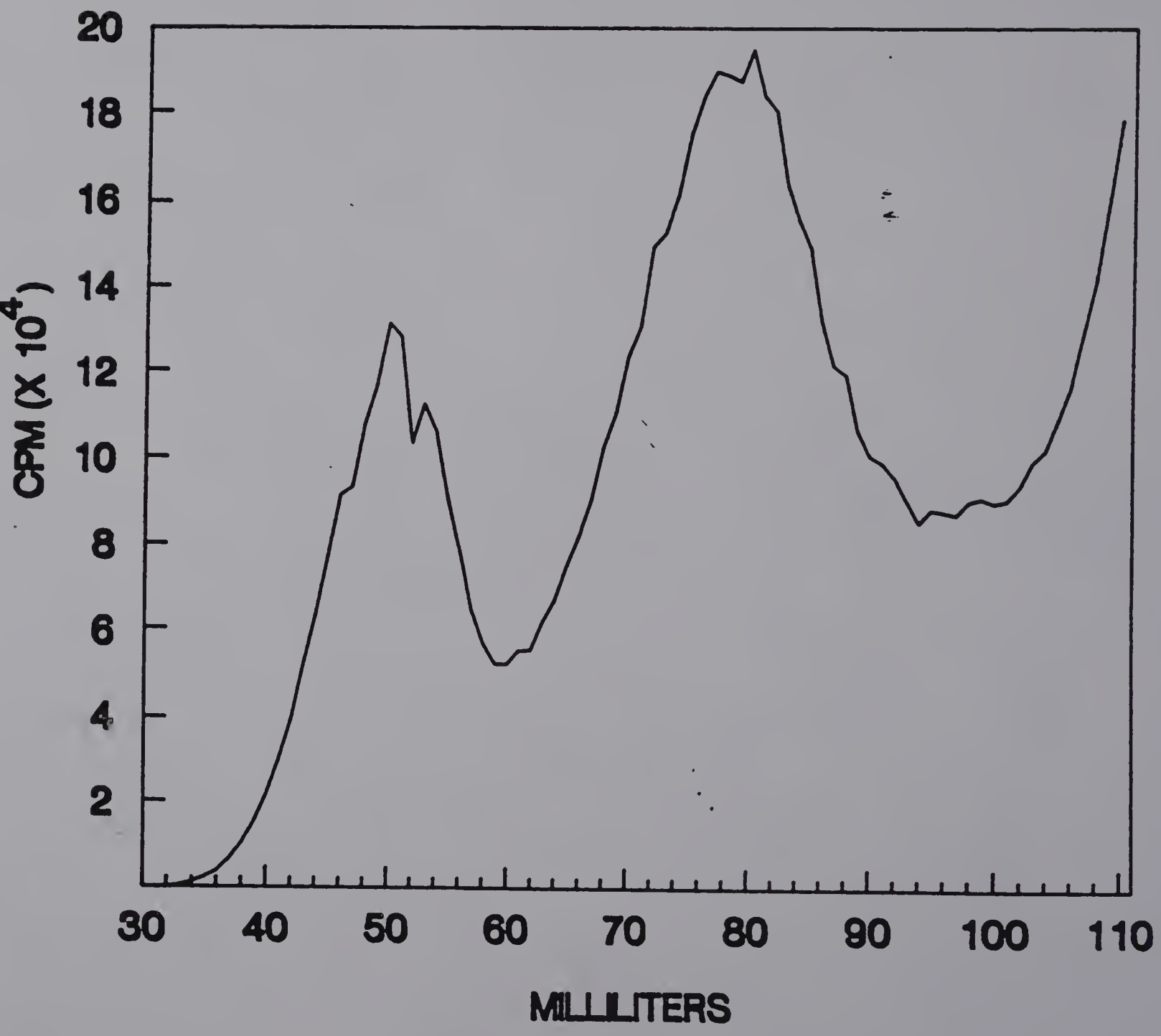

1000
900
800
700
600
500
400
300
200
100
0
CPM
40
50
60
70
80
90
100
110
MILLILITERS

Figure 4. Isolation of ^{125}-I labelled TSST-1 by gel filtration. Crude ^{125}I TSST-1 (Fig. 1, #6) was chromatographed through a 1.5 X 85 cm sephadex G-75 column.

FIGURE 4


20
18
16
14
12
10
8
6
4
2
CPM (X 10^4)
30
40
50
60
70
80
90
100
110
MILLILITERS

the bound protein eluted with a stepwise gradient of increasing molarity and pH. Fractions were monitored for protein concentration by absorbance at 280 nm and for the presence of TSST-1 by double immunodiffusion against the specific rabbit anti-TSST-1 antibody. A typical elution pattern is shown in Figure 5 with the immunoreactive portion shaded. For further purification, immunoreactive fractions were pooled.

The pooled fractions were concentrated and analyzed by SDS-PAGE and the presence of a protein band with a molecular weight corresponding to that of TSST-1 confirmed. The partially purified TSST-1 was iodinated with Bolton-Hunter reagent and reacted with rabbit anti-TSST-1 antibody as described previously and the antibody-antigen complex separated from the unbound label by treatment with fixed Cowan 1 cells. After centrifugation, the supernatant and pellet fractions were separated on a 10 % acrylamide SDS-PAG. An autoradiogram of this gel is shown in Figure 6. The labelled protein preparation contains 2 bands (lane 3) before reaction with the antibody, one of low molecular weight that moved at the tracking dye front and consists of free label and a second with a molecular weight of approximately 22 kDa. The 22 kDa protein band was completely removed from the supernatant portion (lane 2) after reaction with the specific antibody and was only present in the pellet fraction (lane 1). This protein is

Figure 5. CM sephadex ion exchange chromatography of strain MN-8 culture supernatants. Culture supernatants from TSST-1 producing strain MN-8 were absorbed with CM sephadex C-25. Bound protein was eluted with a stepwise gradient of 0.01M $NaHPO_4$ pH 5.8, 0.025M $NaHPO_4$ pH 6.2, and 0.05 $NaHPO_4$ pH 6.6. Five ml fractions were monitored for protein by absorbance at 280 nm., and for immunoreactivity by immunodiffusion (shaded area).

Figure 5

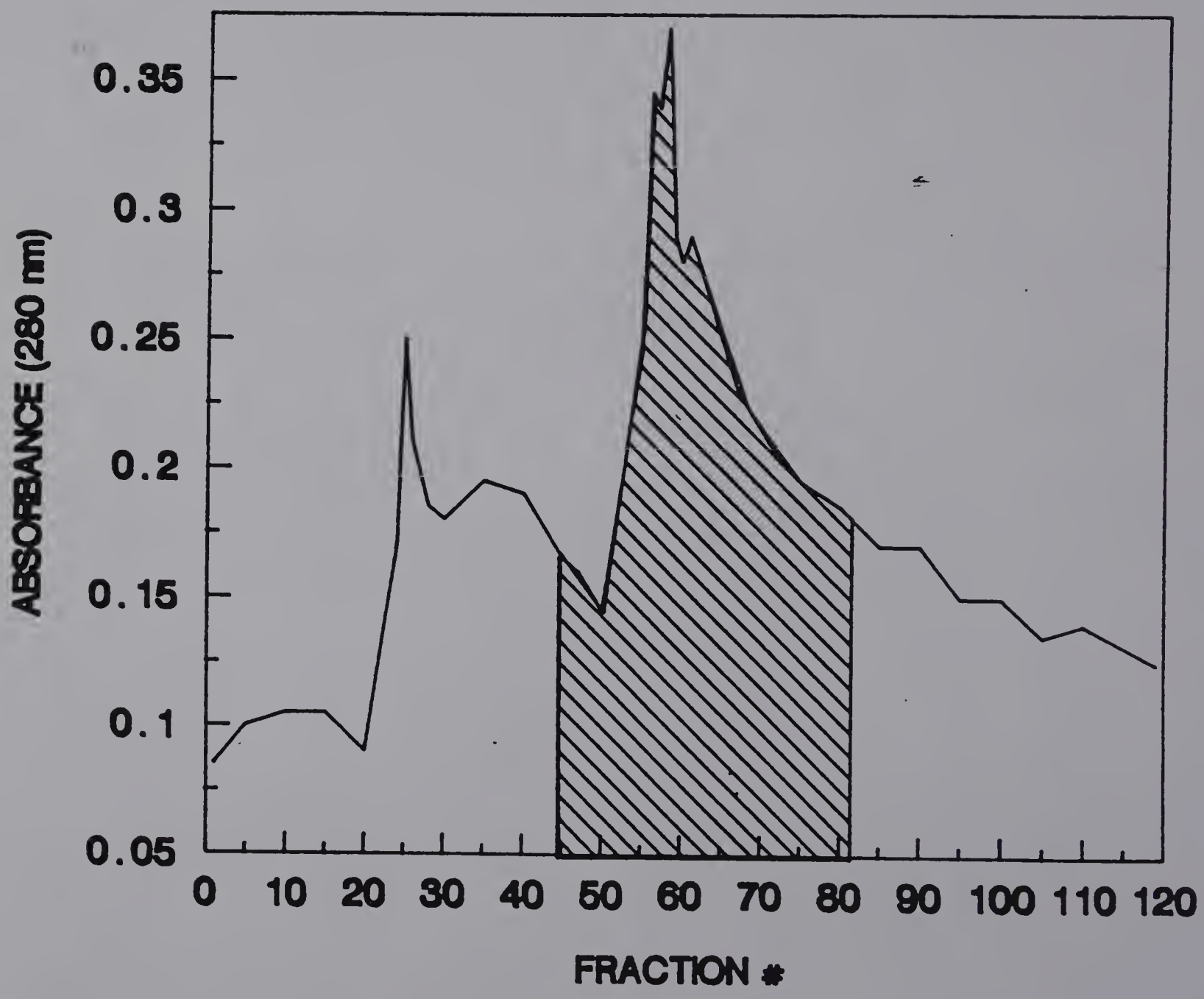
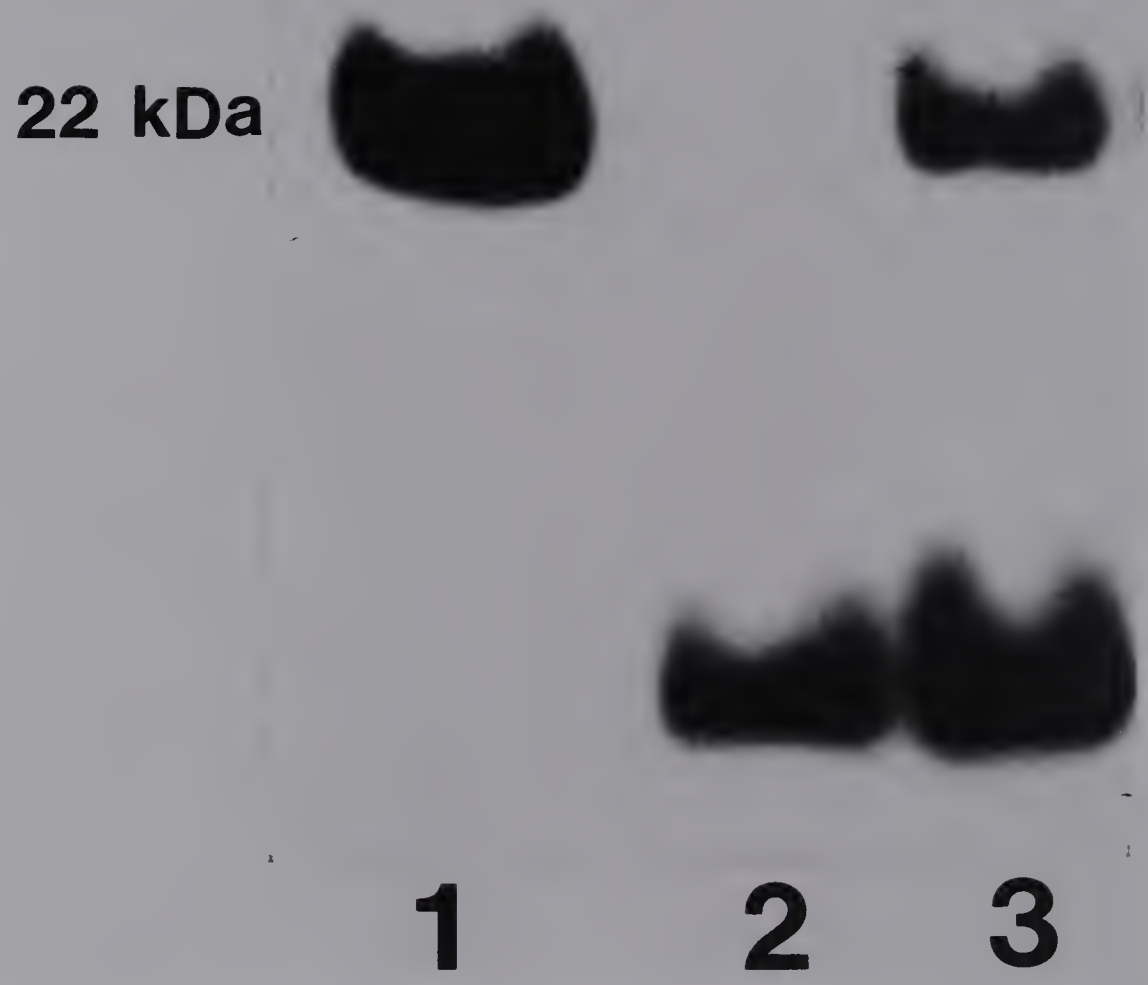

ABSORBANCE (280 nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
10
20
30
40
50
60
70
80
90
100
110
120
FRACTION #

Figure 6. Autoradiogram of ^{125}-I TSST-1 immunoprecipitated with rabbit antiserum. TSST-1 isolated from CM sephadex (Fig. 5) was absorbed with 100 ul rabbit anti-TSST-1 serum and 10% protein-A and separated by SDS-PAGE. Lane 1. TSST-1 absorbed by rabbit antiserum. Lane 2. Supernatant after absorption. Lane 3. Unabsorbed TSST-1.


22 kDa
1
2
3

TSST-1 and no other contaminants are visible in this sample.

The iodinated TSST-1 preparation was also separated by gel filtration over a calibrated Sephadex G-50 column. Two peaks were resolved with the first corresponding to a molecular weight of 18.5 kDa as determined against a calibration curve. The second peak consisted of unbound label (Figure 7). The peak fractions were pooled and chromatographed a second time over the same column (Figure 8), resulting in the elution of a single peak corresponding to TSST-1. This procedure effectively purified TSST-1 but total yield of toxin was low.

Competitive inhibition radioimmunoassay. Purified ^{125}I labelled toxin was used in an RIA to show the production of TSST-1 by specific strains of S. aureus. Toxic shock associated strain MN-8 and non-TSS associated control strain 6538-p, were compared by competitive inhibition RIA for production of TSST-1. Strain MN-8 is a known producer of TSST-1 while strain 6538-p is not. Both MN-8 and 6538-p strains were grown in BHI for 24 hr and the culture supernatants analyzed by serially diluting, adding an aliquot of ^{125}I labelled TSST-1 and specific anti-TSST-1 antiserum. As shown in Figure 9, the culture supernatant from the strain 6538-p did not inhibit binding of the labelled toxin at any dilution tested indicating that it

Figure 7. Isolation of ^{125}I TSST-1 by sephadex G-50 gel filtration. Labelled TSST-1 isolated from CM sephadex C-25 column (Fig. 5) was chromatographed through a 1.5 X 105 cm sephadex G-50 column. Fractions were monitored for total counts in a gamma counter.

Figure 7

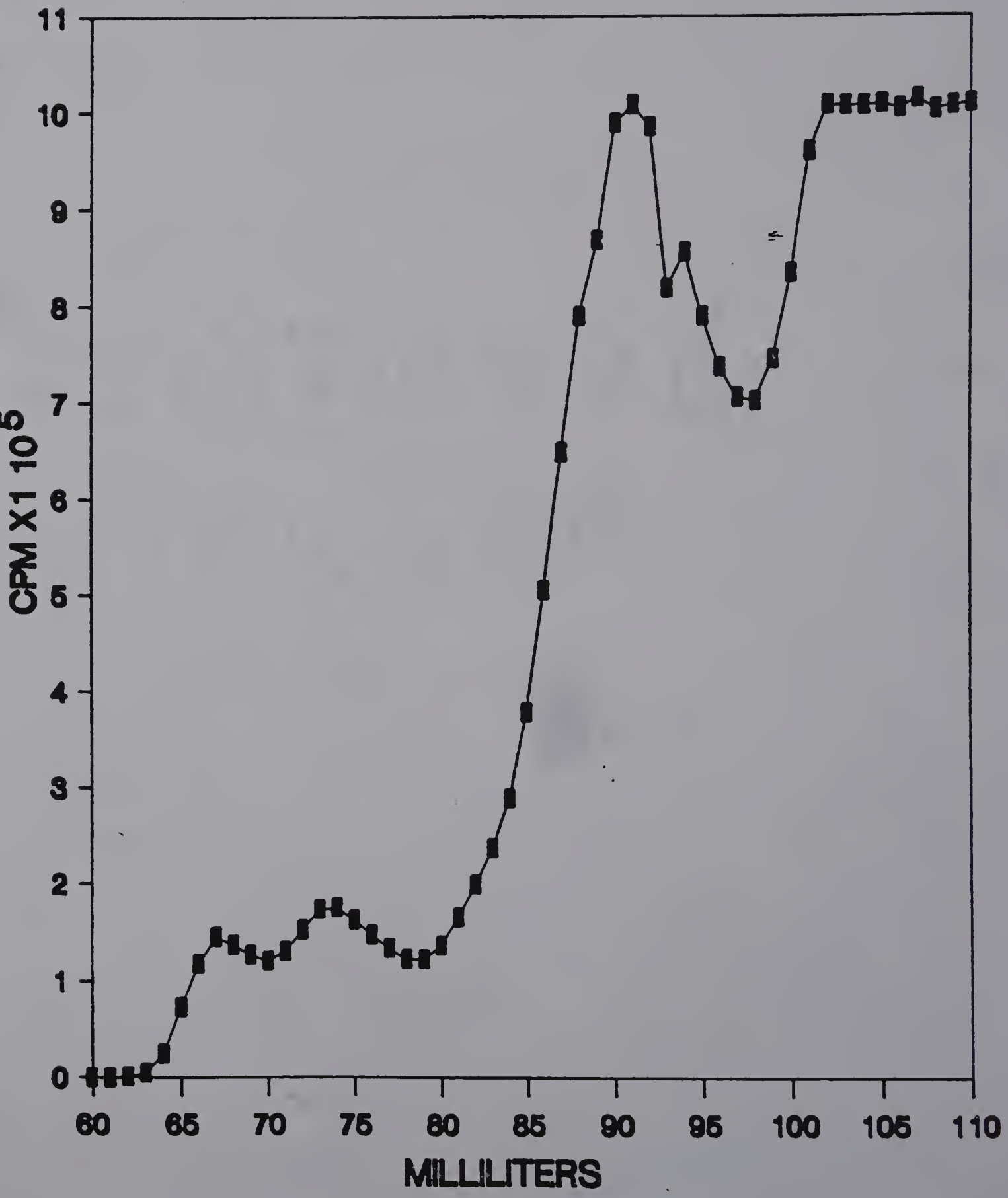

CPM X1 10^5
11
10
9
8
7
6
5
4
3
2
1
0
60
65
70
75
80
85
90
95
100
105
110
MILLILITERS

Figure 8. Isolation of ^{125}I TSST-1 by repeat sephadex G-50 gel filtration. Labelled TSST-1 eluting from the sephadex G-50 (Fig. 7) was rerun over the same column. Fractions were monitored for total counts in a gamma counter.

Figure 8

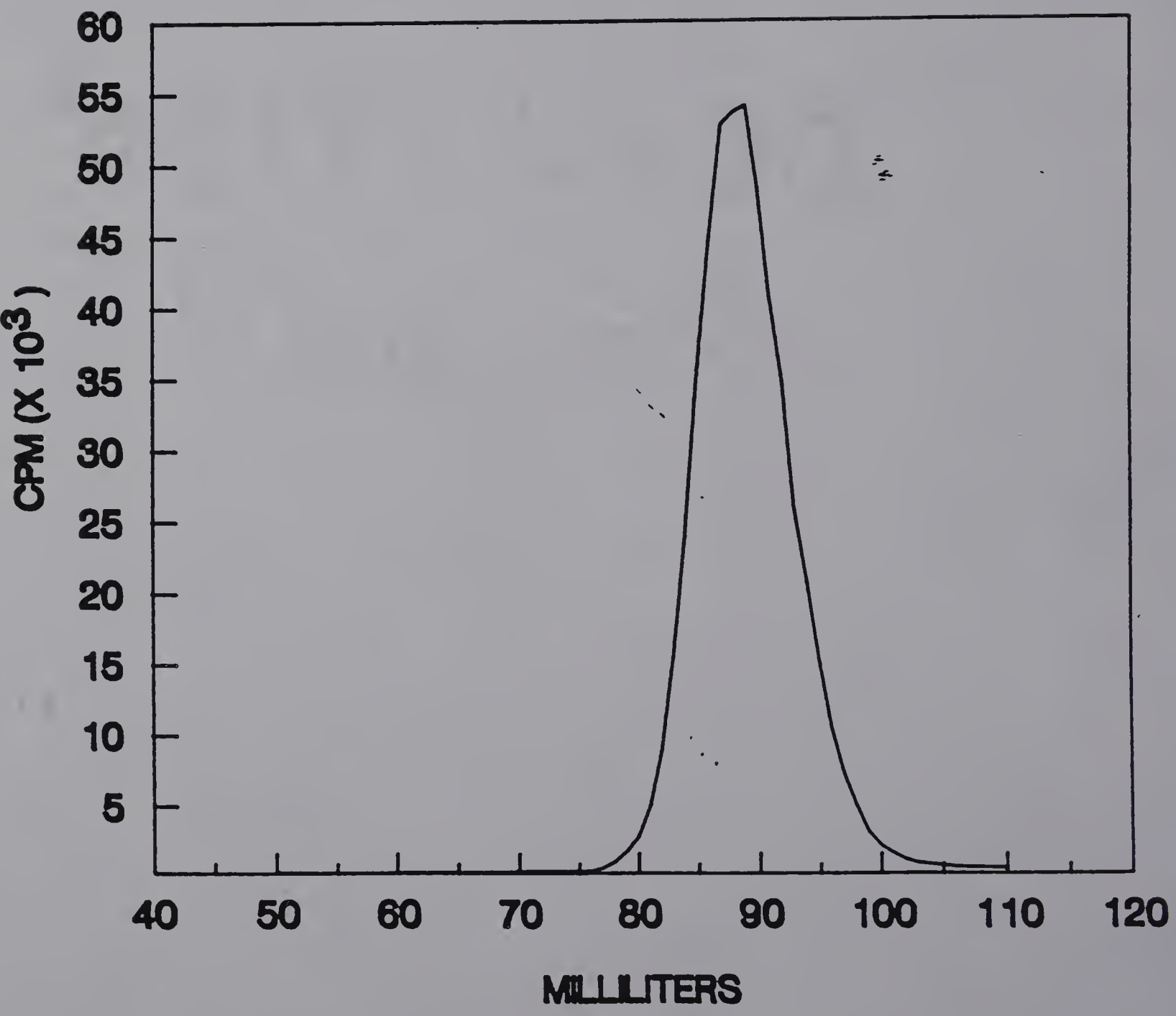
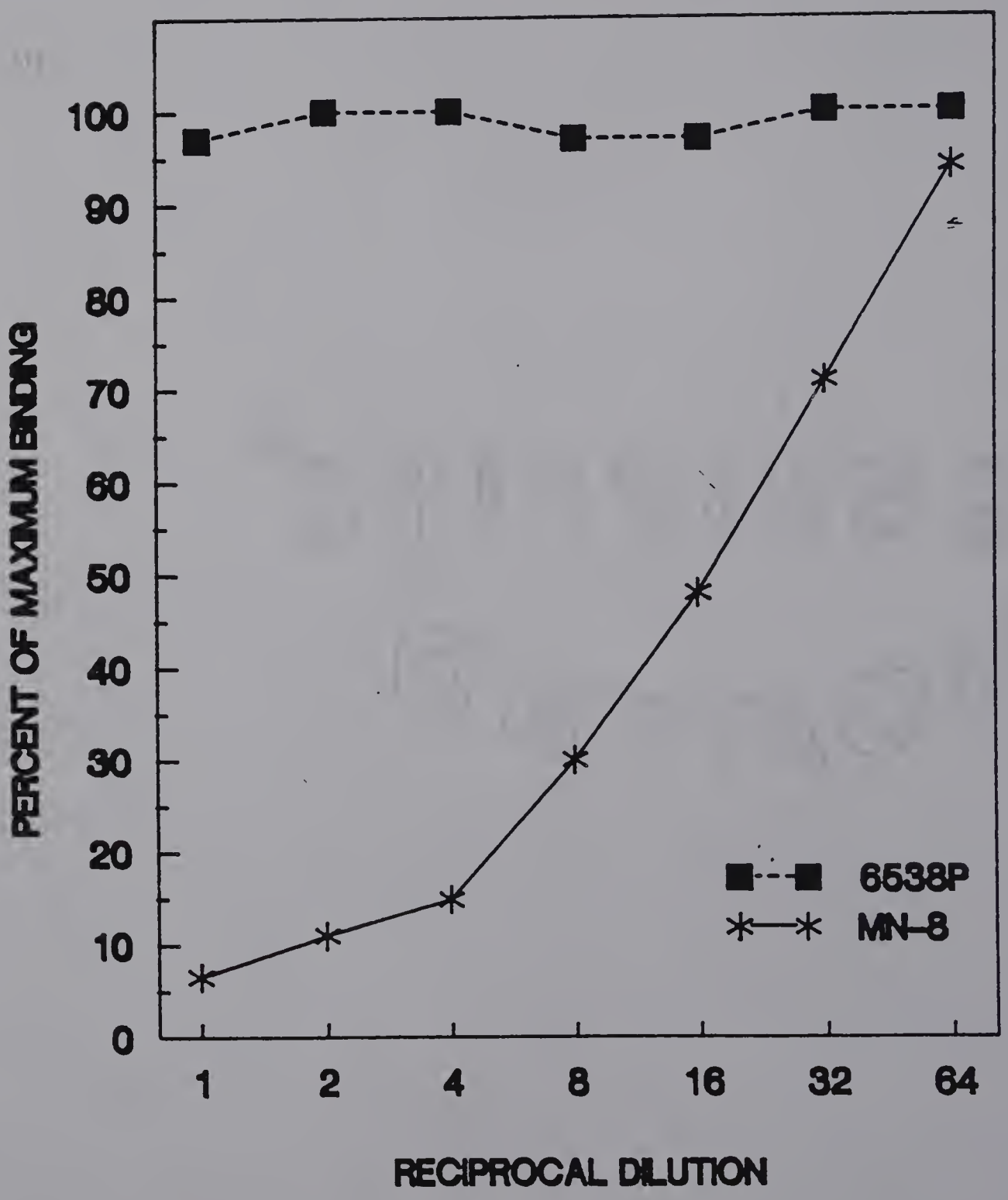

CPM (X 10^3)
60
55
50
45
40
35
30
25
20
15
10
5
40
50
60
70
80
90
100
110
120
MILLILITERS

Figure 9. Competitive RIA inhibition measuring TSST-1 levels in culture supernatants. Fifty ul of culture supernatants from strain MN-8 (—✳—), and strain 6538 (—■—) were incubated with 10^4 cpm ^{125}I TSST-1 and 100 ul rabbit antiserum. The percent of maximum binding was calculated from the total counts bound in the absence of an inhibitor.

Figure 9


PERCENT OF MAXIMUM BINDING
100
90
80
70
60
50
40
30
20
10
0
1
2
4
8
16
32
64
RECIPROCAL DILUTION
6538P
MN-8

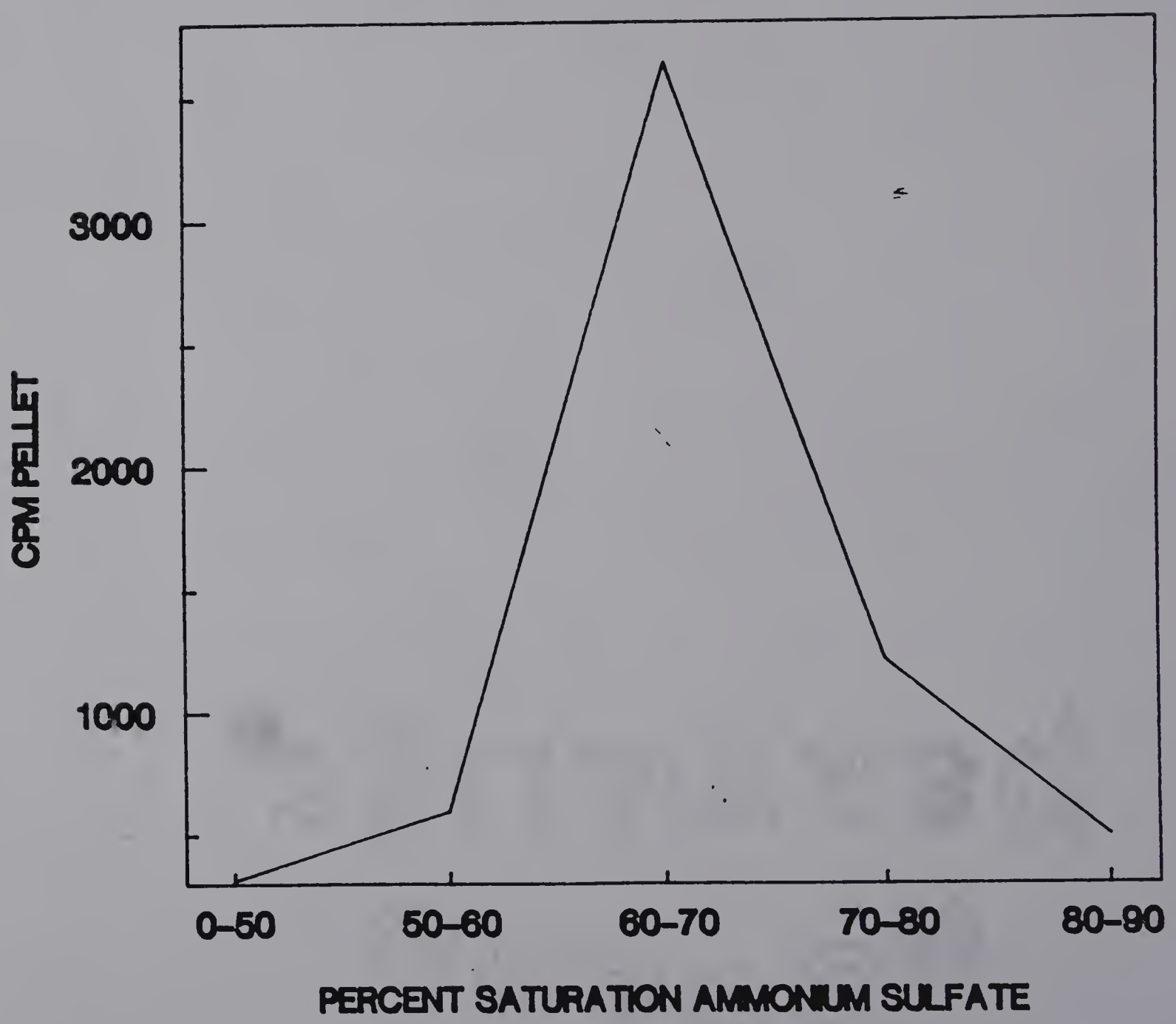
did not produce TSST-1 or other proteins that interfered with the assay. The supernatant from strain MN-8 inhibited binding of the labelled toxin up to a 1/32 dilution.

Further purification of TSST-1. The yield of toxin from adsorption onto CM-sephadex G-25 was low, with less than 20 % of the toxin present in the diluted culture supernatant binding to the resin. A second type of resin, Rexyn 102 (Fisher Scientific Co.,Medford MA) was used for batch adsorption and found to remove all detectable toxin from the supernatant as determined by a competitive inhibition RIA. Prior to adsorption the resin was washed exhaustively with H_20 and equilibrated with 5 mM sodium phosphate buffer at pH 5.6. The toxin was eluted with pH 6.8 buffer containing 0.5 M NaH_2PO_4, 0.5M NaCl. The fractions were monitored for the presence of the toxin by competitive inhibition RIA. Fractions that had inhibitory activity were pooled.

In order to determine the optimal percent saturation of ammonium sulphate for additional purification and concentration, ^{125}I labelled toxin was added to an aliquot of the Rexyn 102 pool. Ammonium sulphate was added and the precipitates from sequential cuts counted. Most of the labelled toxin precipitated out at 60-70% saturation (Figure 10) with a smaller amount at 70-80 %. The

Figure 10. Ammonium sulphate precipitation of TSST-1. Labelled toxin (15000 cpm) was added to an aliquot of a Rexyn 102 isolated toxin pool. Ammonium sulphate was added, the precipitate collected by centrifugation and the counts of sequential cuts determined in a gamma counter.

Figure 10


3000
2000
1000
CPM PELLET
0–50
50–60
60–70
70–80
80–90
PERCENT SATURATION AMMONIUM SULFATE

different cuts were further analyzed by SDS-PAGE. As demonstrated in Figure 11, this confirmed that most of the toxin could be concentrated and purified from the Rexyn 102 eluant using a 60-80 % ammonium sulphate cut. For additional large batch purifications of TSST-1, a 60-80% cut was routinely used.

The preparation of partially purified toxin was further purified by gel filtration on a Sephadex G-50 column (Figure 12). The protein concentration of the fractions was monitored by determining absorbance at 280 nm. Three protein peaks were eluted with the second peak consisting of TSST-1 as confirmed by SDS-PAGE analysis. Fractions 82-93 were pooled, concentrated and passed through the Sephadex G-50 column a second time (Figure 13) eluting as single peak. The fractions were analyzed by SDS-PAGE and then pooled and concentrated. These fractions consisted of a single protein band with a molecular weight of 22 kDa (Figure 14). The purified TSST-1 was also analyzed by isoelectric focusing and was found to have an isoelectric point of pH 7.0 (Figure 15).

Production of rabbit anti-TSST-1 antiserum. When the preparation of TSST-1 (Figure 13) was confirmed by immunoreactivity with rabbit monospecific anti-TSST-1 antiserum, an effort was made to produce additional

Figure 11. SDS-PAGE analysis of ammonium sulphate cuts of TSST-1. Protein bands were resolved by 10% SDS-PAGE and were stained with coomassie blue. Lane 1. Purified TSST-1. Lane 2. 10 ug of a 60%-80% ammonium sulphate cut of Rexyn 102 purified toxin pool. Lane 3. 20 ug. Lane 4. 40 ug. Lane 5. Unfractionated Rexyn 102 toxin pool.


66
45
36
29
24
20.1
14.2
1
2
3
4
5

Figure 12. Isolation of TSST-1 by sephadex G-50 gel filtration. A Rexyn 102 purified toxin pool was precipitated with a 60%-80% ammonium sulphate cut. The precipitate was desalted by dialysis before chromatographing through a 1.5 X 85 cm. sephadex column. Protein eluting from the column was monitored by absorbance at 280 nm.

Figure 12

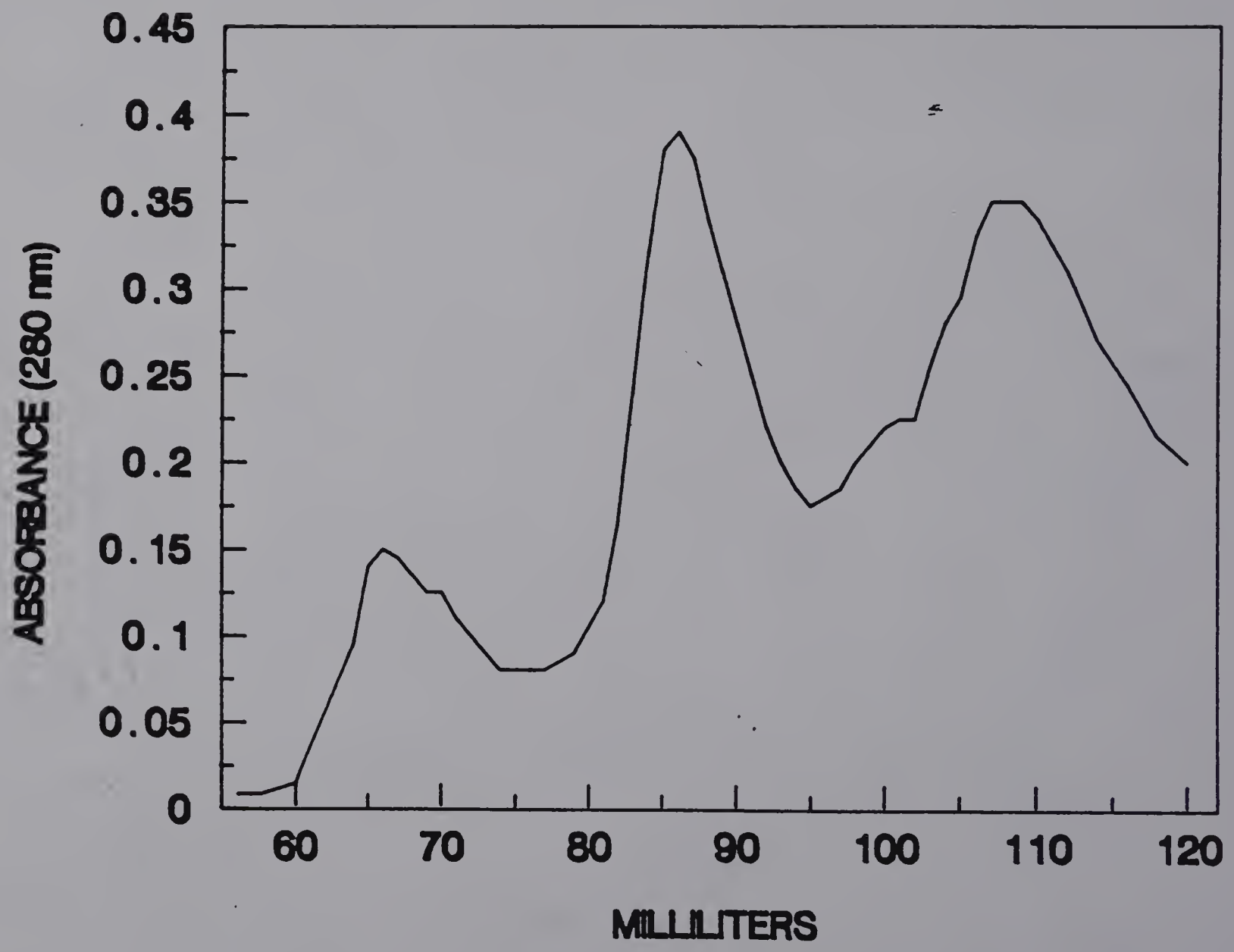

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ABSORBANCE (280 nm)
60
70
80
90
100
110
120
MILLILITERS

Figure 13. Purification of TSST-1 by sephadex G-50 gel filtration. TSST-1 eluting from a sephadex G-50 column (Fig. 12, peak 2) was rerun over the same 1.5 X 85 cm sephadex G-50 column. Protein eluting from the column was monitored by absorbance at 280 nm.

Figure 13

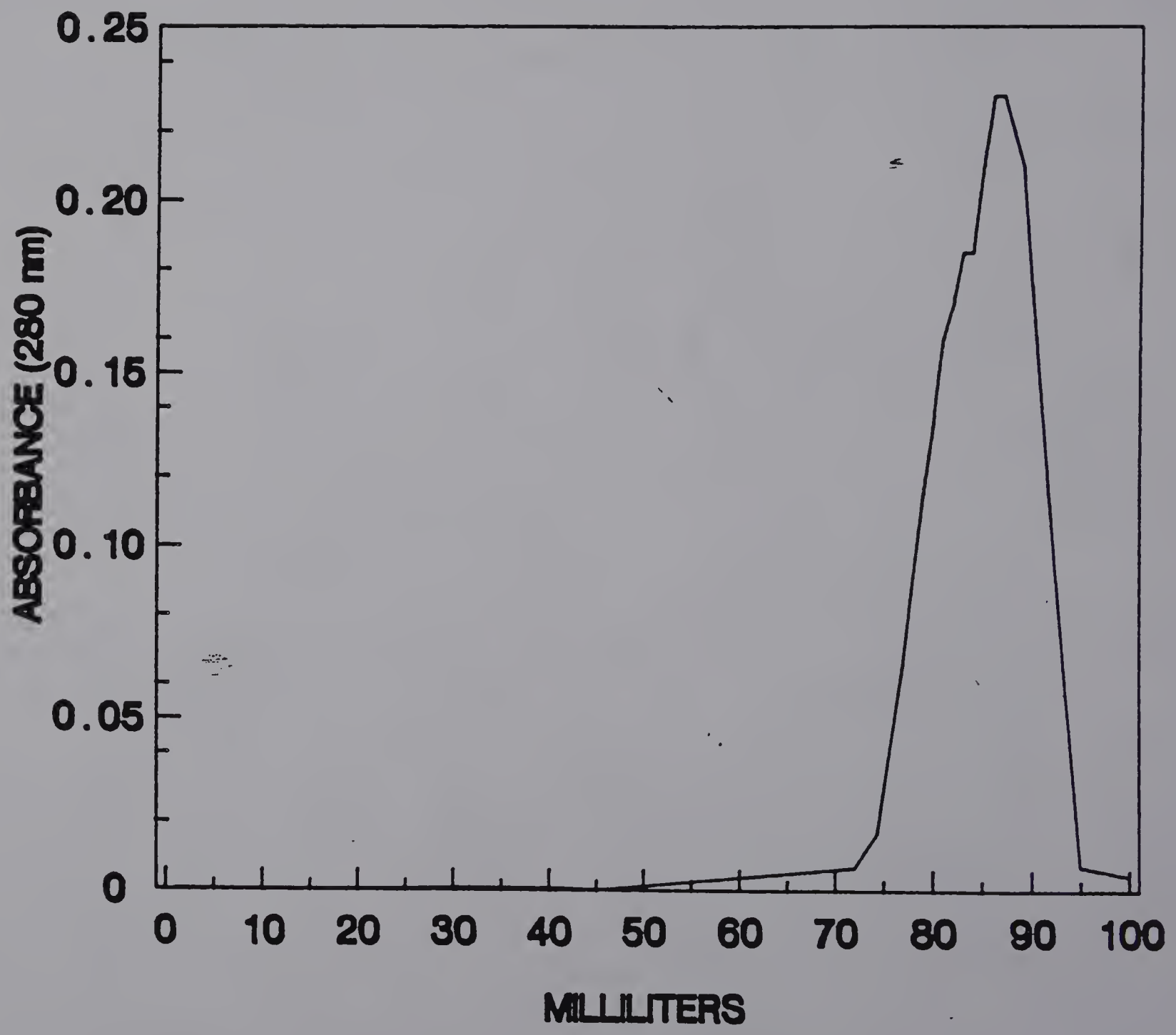

0.25
0.20
0.15
0.10
0.05
0
ABSORBANCE (280 nm)
0
10
20
30
40
50
60
70
80
90
100
MILLILITERS

Figure 14. SDS-PAGE analysis of sephadex G-50 purified TSST-1. Twenty ug of protein from fractions eluting off the sephadex G-50 column (Fig. 13) were analyzed by 10% SDS-PAGE. Protein bands were stained with coomassie blue. Lane 1. Purified TSST-1 standard. Lane 2. Fraction 83. Lane 3. Fraction 84. Lane 4. Fraction 85. Lane 5. Fraction 86. Lane 6. Fraction 87. Lane 7. Fraction 88.

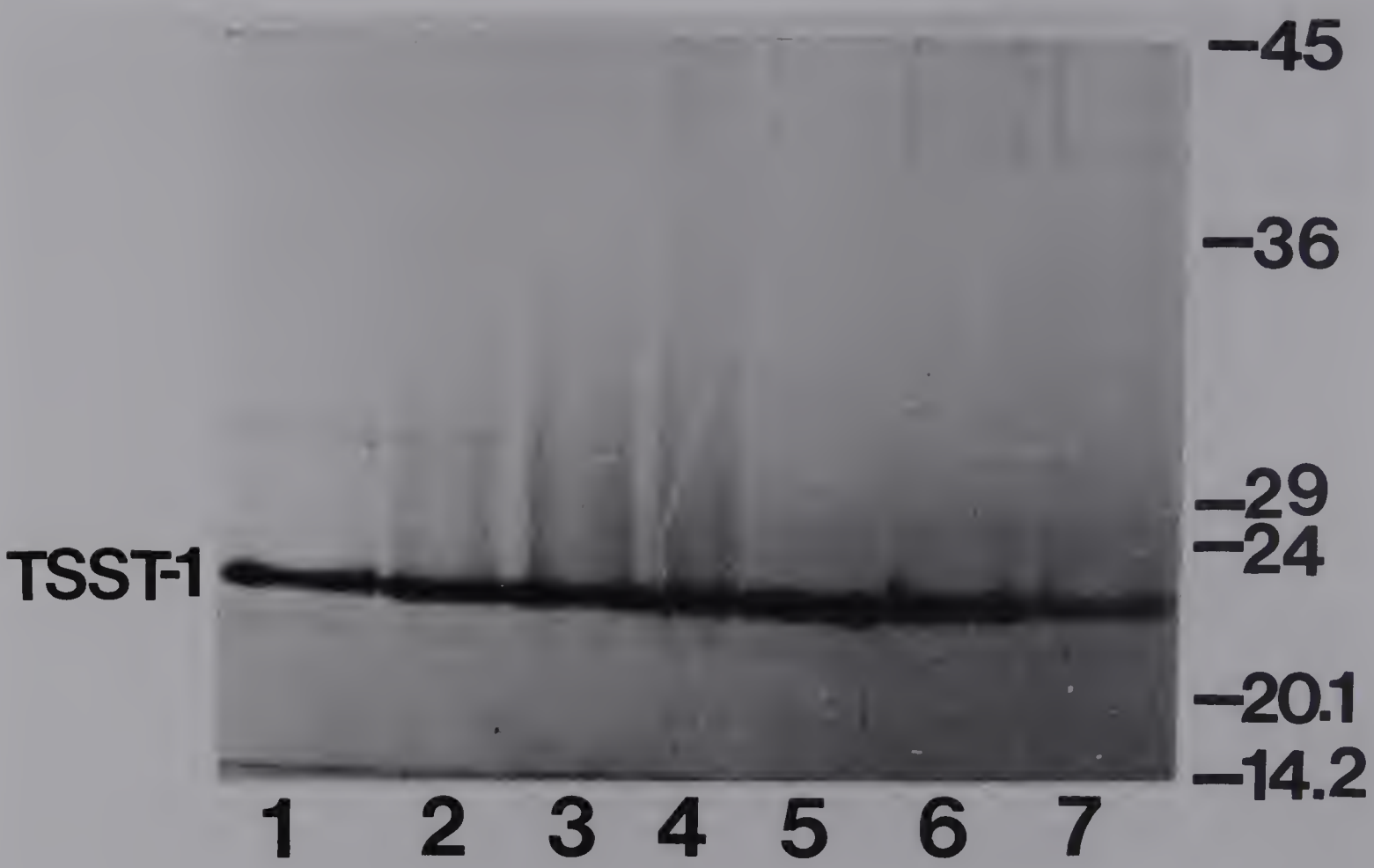
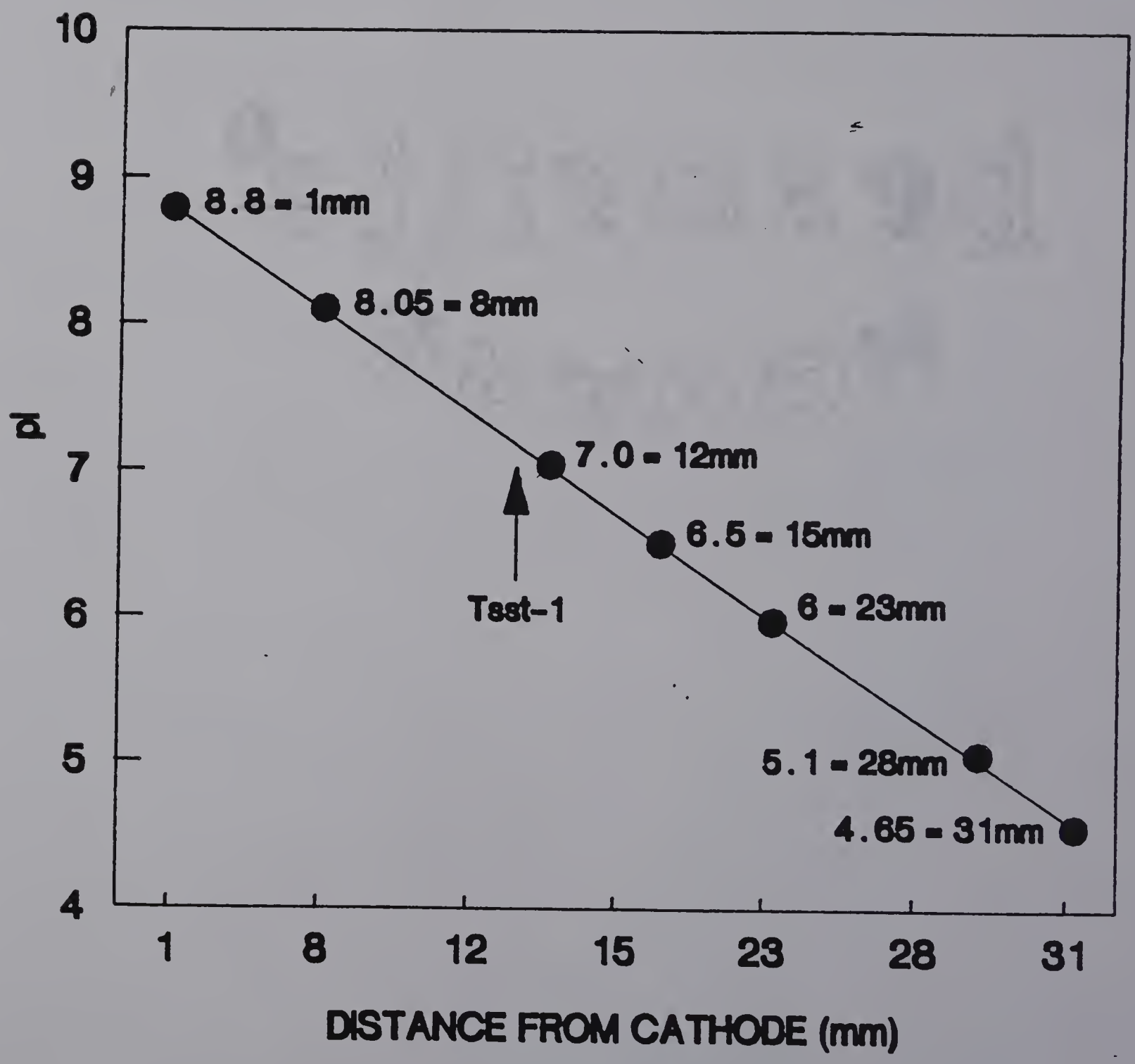

TSST-1
45
36
29
24
20.1
14.2
1 2 3 4 5 6 7

Figure 15. Isoelectric focusing gel analysis of TSST-1. Purified TSST-1 (Fig. 13) was focused on a pH 3-8 gradient gel. The pI of the toxin was determined from a standard curve using alpha-chymotrypsin (pI 8.8), whale myoglobin (pI 8.05), equine myoglobin (pI 7.0), human carbonic anhydrase (pI 6.5), bovine carbonic anhydrase (pI 6.0),beta-lactoglobulin B (pI 5.1) and phycocyanin (pI 4.65) as standards.

Figure 15


10
9
8
7
6
5
4
α
8.8 = 1mm
8.05 = 8mm
7.0 = 12mm
6.5 = 15mm
6 = 23mm
5.1 = 28mm
4.65 = 31mm
Test-1
1
8
12
15
23
28
31
DISTANCE FROM CATHODE (mm)

antiserum in a New Zealand white rabbit. TSST-1 was concentrated by precipitation with cold EtOH, mixed with BSA, and polymerized with ethyl chloroformate (Mahfouz et al. 1980). The resulting precipitate was divided into three aliquots. Each aliquot was dispersed with a tissue grinder before injection, centrifuged, resuspended in H_2O and mixed with an equal amount of Freund's complete adjuvant. The rabbit was inoculated subcutaneously every 2 weeks for a total of 3 times and was bled 2 weeks after the last injection. The rabbit did not develop detectable antibodies against the polymerized toxin when tested by RIA against labelled TSST-1.

Production of goat anti-TSST-1 antibody. Approximately 200 ug of TSST-1 from the pooled peak after sephadex G-50 gel chromatography (Figure 12) was separated on a non-denaturing polyacrylamide gel. The outside lanes were cut off, stained rapidly and realigned with the center portion of the gel. The area corresponding to the TSST-1 band was excised from the gel. The TSST-1 portion of the gel was ground with a tissue grinder, suspended in a minimal amount of water, mixed with Freund's complete adjuvant and injected intramuscularly and subcutaneously into the hindquarters of a goat. This procedure was repeated 3 weeks after the first injection and the goat was bled one week after that. Immunoreactivity of the goat serum was visible by double immunodiffusion against purified TSST-1

and showed lines of complete identity with the monospecific rabbit anti-TSST-1 (Figure 16). This reaction pattern indicates that both antisera are recognizing the same protein, TSST-1, as the antigen.

Molecular shift of TSST-1 with goat and rabbit antisera. Both goat and rabbit IgG were purified from serum by precipitation with Na_2SO_4 and gel filtration through a Sepharose 6B-Cl column. Purified goat and rabbit anti-TSST-1 IgG were reacted with ^{125}I labelled TSST-1 and then separated by gel filtration over a sephacryl S-200 column. Purified normal IgG from each species was run as controls. Both the goat and rabbit antibodies prepared against TSST-1 shifted the labelled protein from where it normally elutes with a peak at fraction 85 to a peak at the void volume of the column. Normal IgG from both species did not react with the labelled TSST-1 at all. This again confirmed the similarity in immunoreactivity of the two immunoglobulin preparations (Figure 17A,B).

Affinity purification of TSST-1. Using a ligand of specific goat anti-TSST-1 IgG, an affinity column was prepared by covalently binding IgG with affigel-10 support. Culture supernatant from strain MN-8 was concentrated by

Figure 16. Double immunodiffusion of antisera against TSST-1. Wells cut in a 1% agarose gel were loaded with five ul reagent and incubated 48 hours at 5°C. Precipitin lines were stained with coomassie blue.

A) Rabbit anti TSST-1.
B) goat anti-TSST-1.
C) TSST-1.

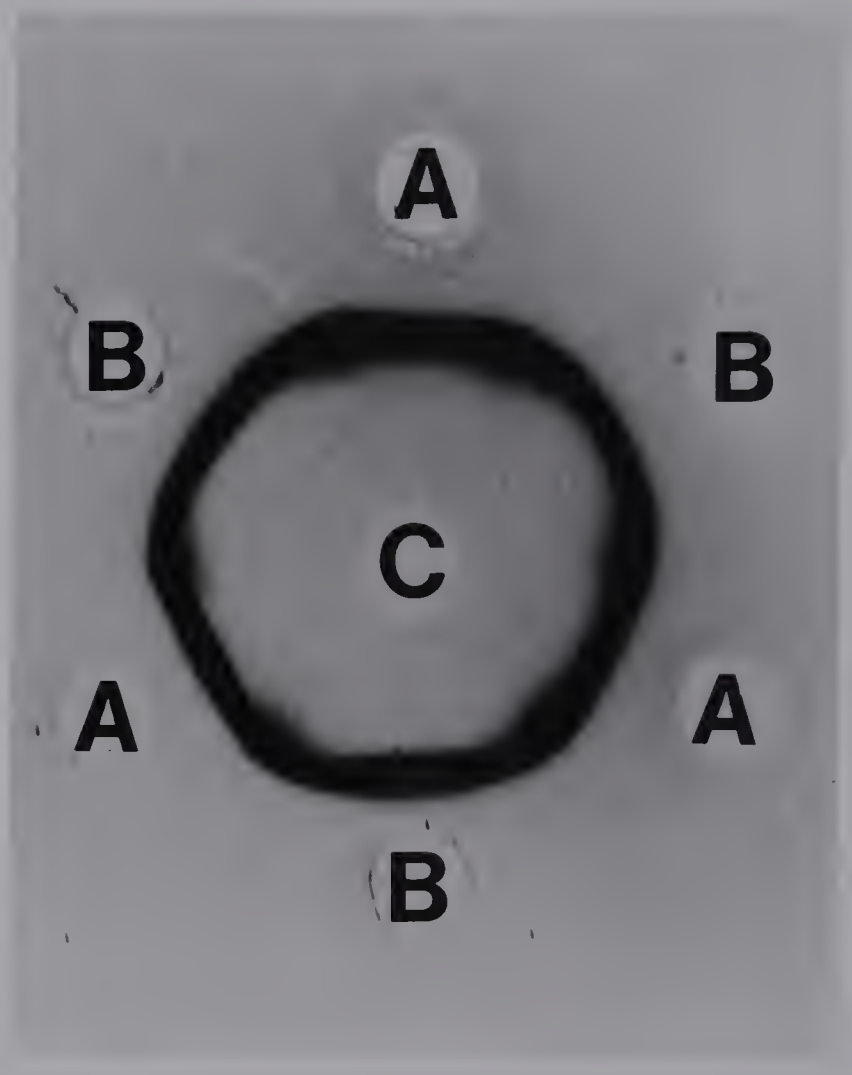
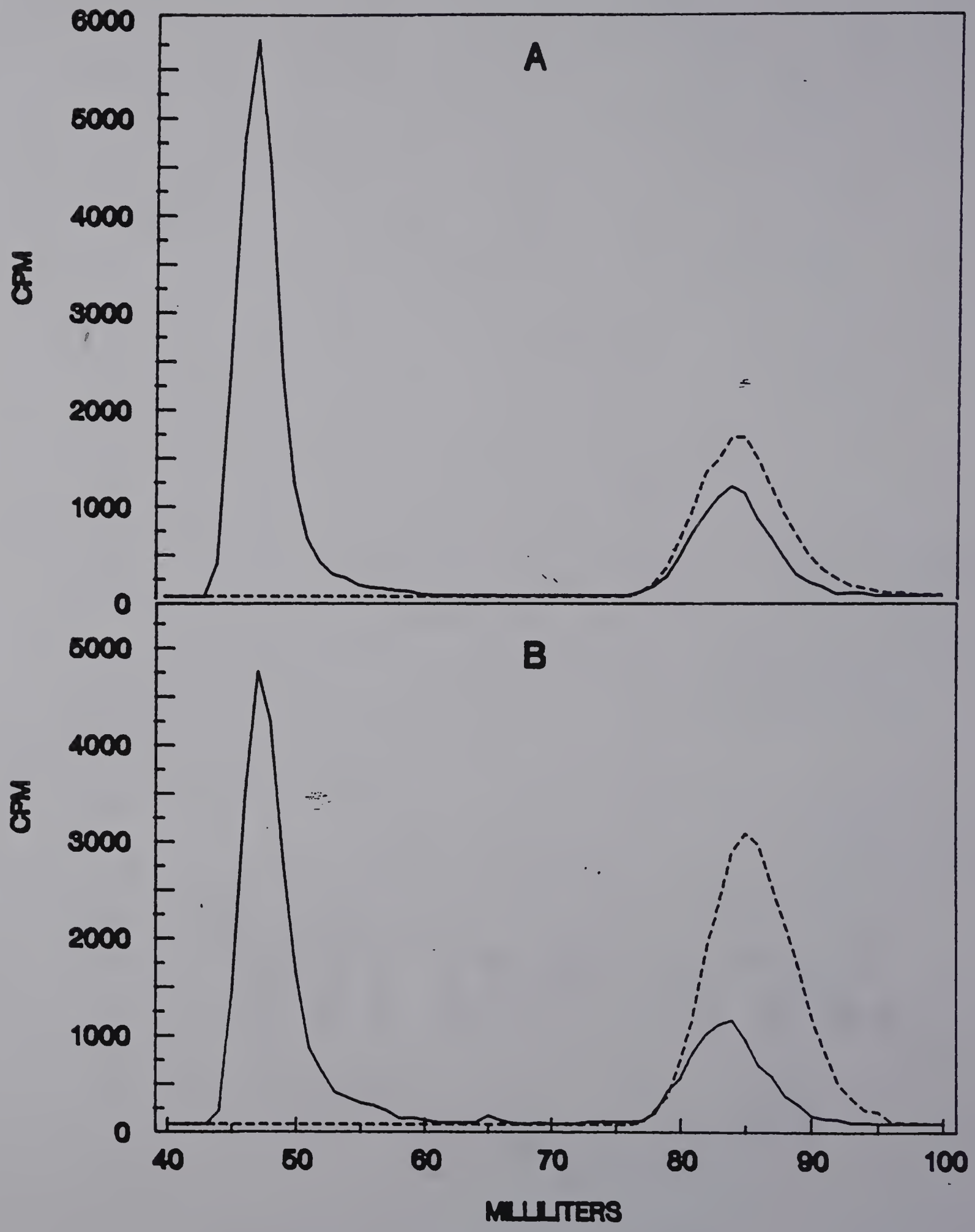

A
B
B
C
A
A
B

Figure 17. Molecular shift chromatography of TSST-1 with goat and rabbit antisera. ^{125}I TSST-1 was reacted with A) rabbit anti-TSST-1 IgG (——) or non-immune IgG (- - -); and B) goat anti-TSST-1 IgG (——) or non-immune IgG (- - -). The reaction mixtures were chromatographed through a sephacryl S-200 (1.5 X 85 cm) column and the fractions were monitored for total counts in a gamma counter.

Figure 17


A
B
CPM
6000
5000
4000
3000
2000
1000
0
40
50
60
70
80
90
100
MILLILITERS

adsorption on Rexyn 102 ion exchange resin, eluted and dialyzed against 0.01 M Tris-HCl, pH 7.5. The crude protein preparation was adsorbed onto the affinity column and rinsed with additional buffer until the absorbance at 280 nm was zero. Protein on the column was eluted with 0.1 M glycine, pH 2.5. The fractions were monitored for protein by absorbance at 280 nm (Figure 18). A single peak eluting from the affinity column was analyzed by SDS-PAGE and found to be homogeneous TSST-1 (Figure 19).

Figure 18. Affinity purification of TSST-1. Toxin was partially purified from culture supernatants by ion exchange chromatography on a Rexyn 102 column. The crude protein preparation was absorbed onto a goat anti-TSST-1 affigel-10 affinity column. Toxin bound to the column was eluted with 0.1M glycine pH 2.5. Fractions eluting from the column were monitored for pH (---) and absorbance at 280 nm (——).

Figure 18

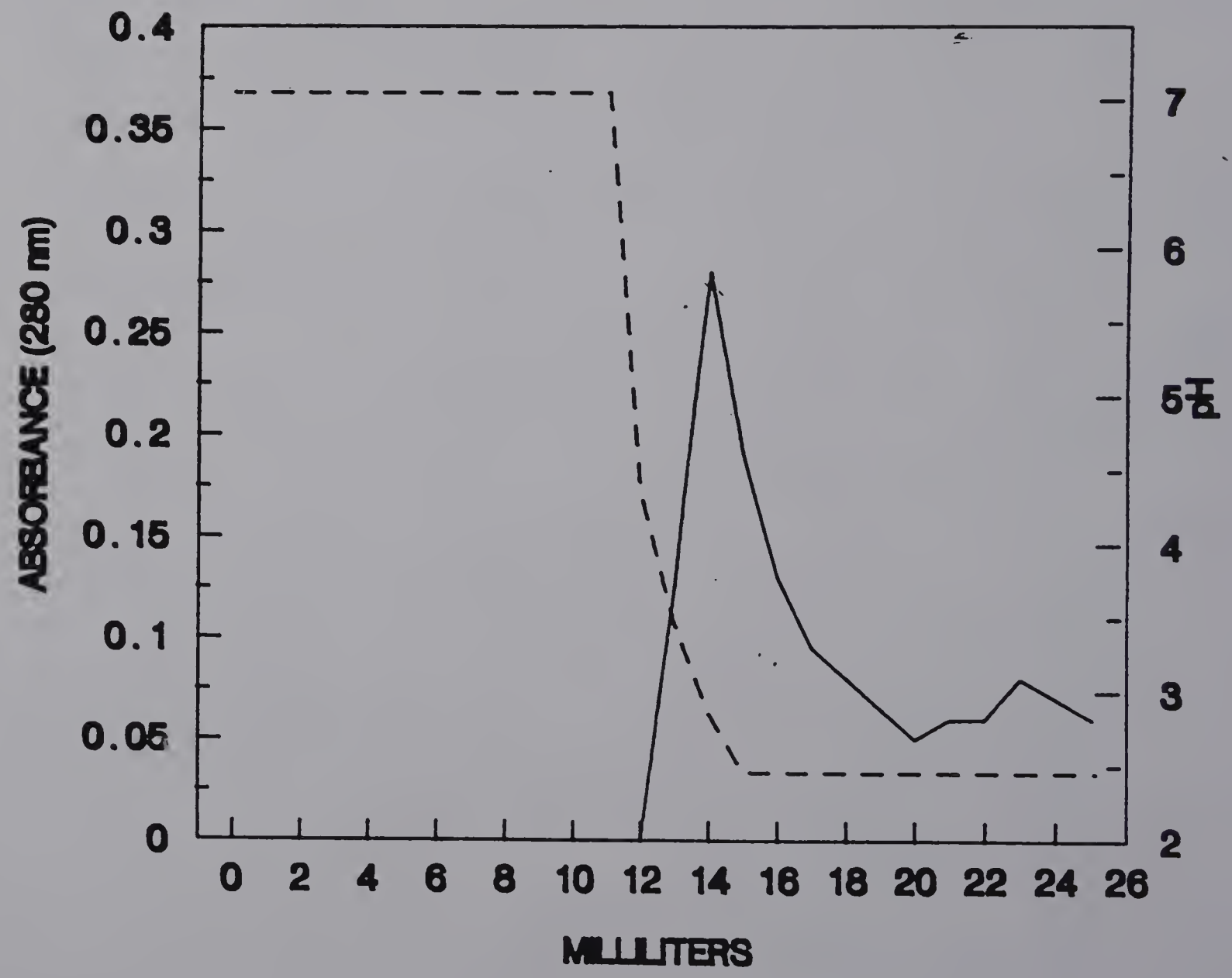
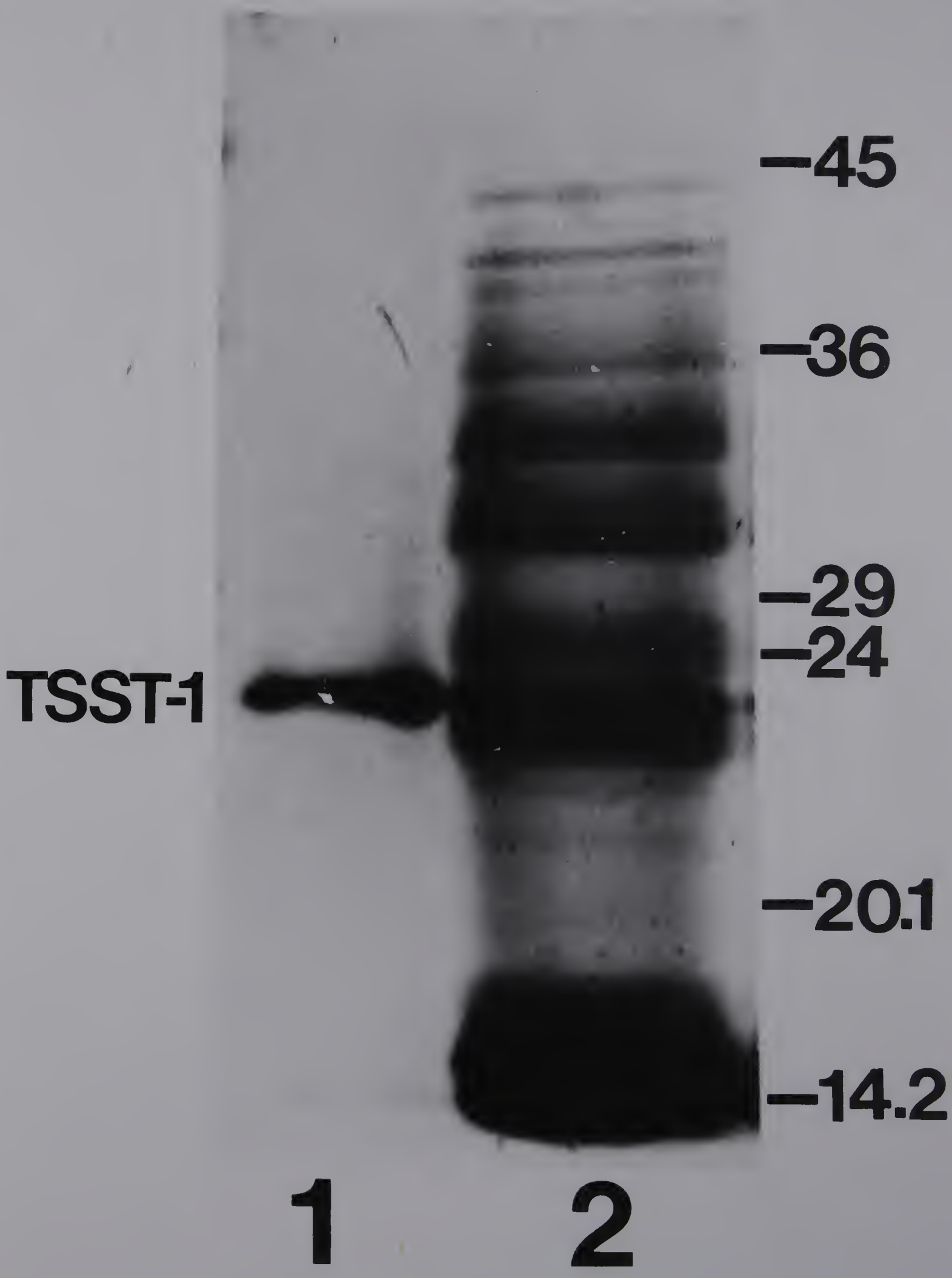

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ABSORBANCE (280 nm)
7
6
5
4
3
2
0 2 4 6 8 10 12 14 16 18 20 22 24 26
MILLILITERS

Figure 19. SDS-PAGE analysis of affinity purified TSST-1. Lane 1. Affinity purified TSST-1. Lane 2. Crude protein eluted from Rexyn 102 ion exchange column.


45
36
29
24
20.1
14.2
TSST-1
1
2

Interaction of TSS Associated S. aureus with Tampon Fibers

Survival of S. aureus on tampon fibers. When epidemiological studies first uncovered the relationship of tampons to TSS, one hypothesis was that the tampons themselves were intrinsically contaminated with S. aureus and acted as fomites introducing the organism into the vagina (Broome et al.,1982). In deference to this theory, a TSS-associated and a control strain of S. aureus were inoculated onto 3 different types of dry tampon fibers. After inoculation the fibers were allowed to dry at room temperature and were sampled for viable organisms over time.

As shown in Figure 20, the TSS associated strain 189 was recoverable from all three types of fibers for at least 80 days. The cfu recovered from each fiber piece ranged from 10^1 to 10^3 with no noticeable difference in survival on any of the three fibers. The non-TSS associated strain ATCC# 6538-p showed a fiber related difference in persistence on the different fibers (Figure 21). Starting with initial counts of 1×10^7 cfu per fiber piece, after 78 days 10^4-10^5 cfu were still detected on the Super Plus tampon fiber (Tampax) composed of polyacrylate rayon (PAR). In contrast, the recoverable cfu on the Original Regular and Slender Regular (cotton and a mixture of rayon and carboxymethylcellulose respectively) were reduced to less

Figure 20. Recovery of S. aureus strain 189 from tampon fibers. Original Plus (OR), Slender Regular (SR), and Super Plus (P) tampon fibers were inoculated with 10^7 cfu of S. aureus strain 189. After drying the fibers were stored in petri dishes at ambient temperature. Organisms were serially diluted in TSB followed by enumeration on TSA plates using a smear plate technique.

Figure 20

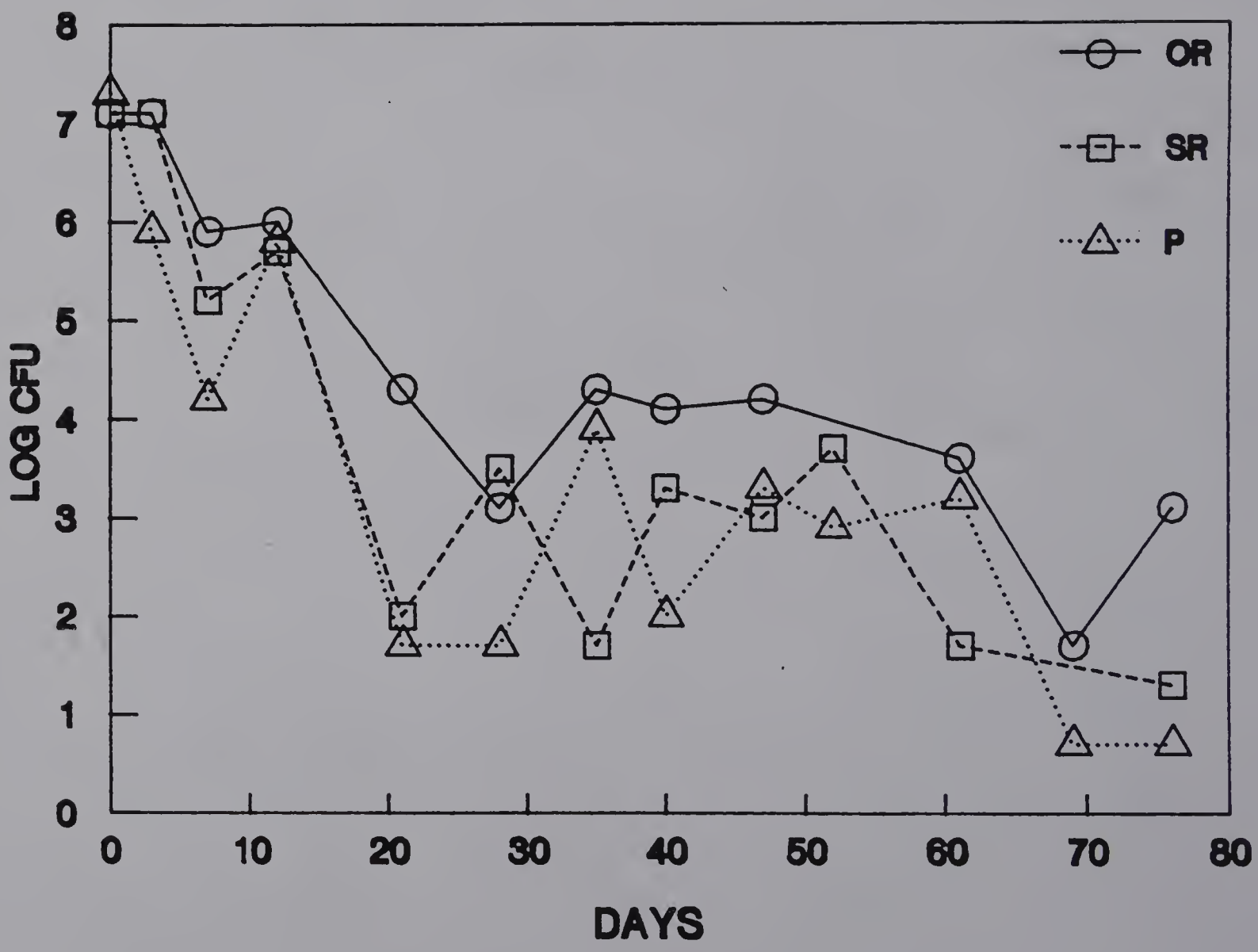
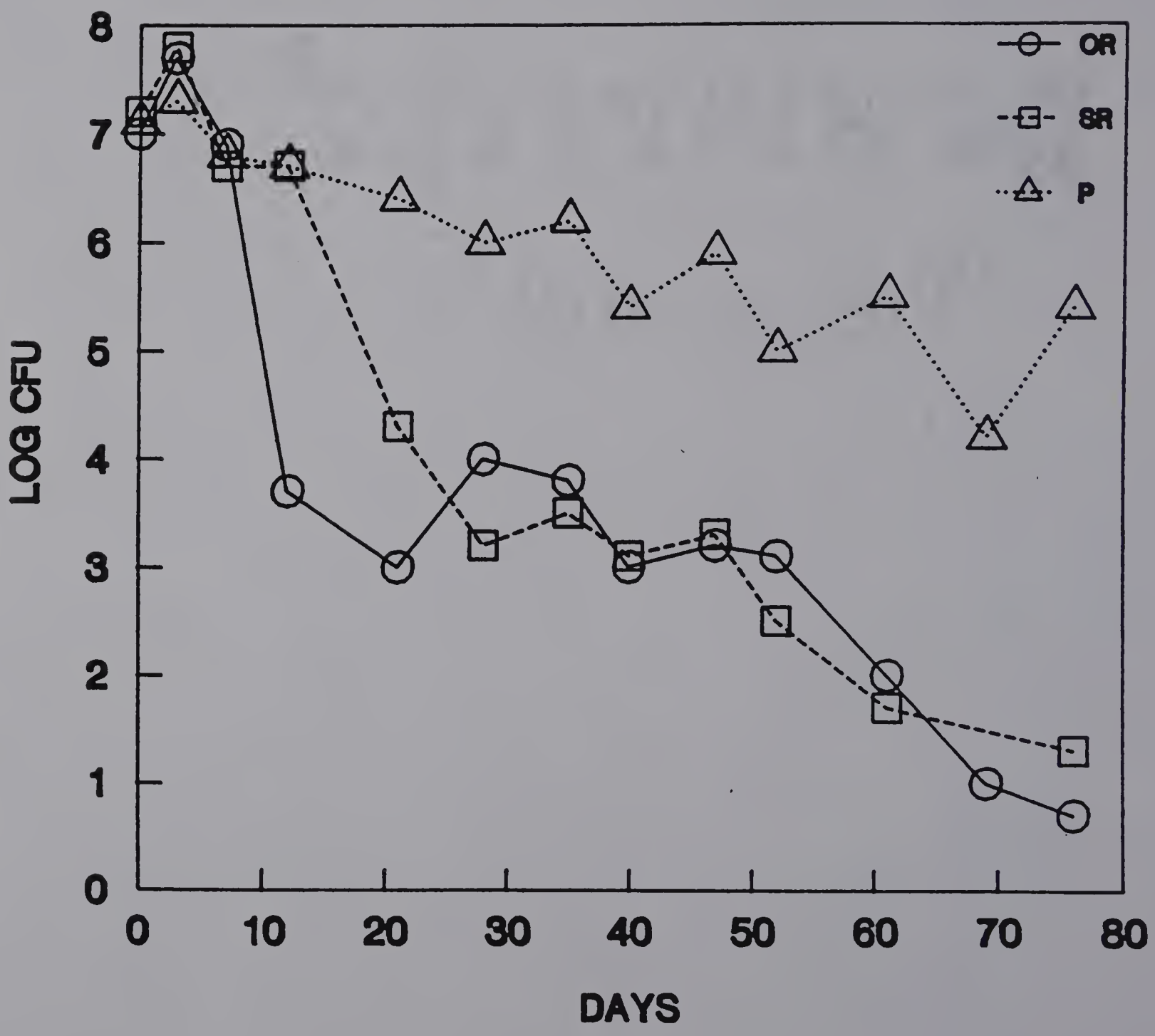


Figure 21. Recovery of S. aureus strain 6538-p from tampon fibers. Original Plus (OR), Slender Regular (SR), and Super Plus (P) tampon fibers were inoculated with 10^7 cfu of S. aureus strain 6538-p. After drying, the fibers were stored in petri dishes at ambient temperature until tested. Organisms were serially diluted in TSB followed by enumeration on TSA plates using a smear plate technique.

Figure 21



than 10^2 cfu on each piece of fiber.

Magnesium chelating ability of tampon fibers. Certain tampon fibers have the ability to chelate magnesium ions (Mg^{++}) and consequently can have an impact on Mg^{++} concentration in solutions. As demonstrated by Mills et al. (1985) the concentration of magnesium can have an effect on the production of TSST-1 by TSS associated strains of S. aureus. In light of this, the ability of specific fibers to chelate magnesium from aqueous solutions and culture medium (BHI) was examined. One gram of each fiber was placed inside dialysis tubing with a retention limit of 1000 kDa and moistened with 10 ml of H_2O. In the case of carboxymethylcellulose (CMC) only 0.25 g/ 10 ml was used because of its viscous and highly hydroscopic nature. Each fiber was dialyzed for one week against a solution of 100 ppm magnesium ($MgCl_2$). The solution outside the dialysis bag was analyzed for residual magnesium. As presented in Table 1, cotton, rayon and washed polyester foam (PEF, Rely tampon) did not bind significant Mg^{++} from the solution while CMC and PAR removed almost all of the Mg^{++} leaving little outside the dialysis bag. The unwashed PEF which still contained a small portion of CMC bound more Mg^{++}/g fiber than the washed PEF and substantially less than the CMC.

Table 1. Magnesium binding ability of fiber in aqueous solution.

Fiber	ug Mg^{++} bound/g fiber colorimetric	AA*
Rely		
unwashed PEF	1070	-
washed PEF	240	240
CMC	10570	8360
PAR	4790	4710
Cotton	240	-
Rayon	240	-

*Atomic absorption spectrophotometer

When BHI was extracted with different types of tampon fibers, it was noticed that Mg++ was selectively removed by both polyacrylate rayon and Rely tampons. Since Rely tampons are composed of two types of fiber, PEF and CMC, an effort was made to differentiate between the Mg++ binding ability of the PEF and CMC in comparison to PAR, cotton and rayon.

Since it was likely that the protein and salts in a culture medium such as BHI, would affect the divalent cation binding ability of the fibers and consequently affect the production of TSST-1, the removal of magnesium from BHI by fibers was examined. Different fibers were

added to BHI to a concentration of 10 % (W:V) and incubated for 1 hr. The BHI was pressed from the fibers and analyzed for Mg^{++} concentration. The results as presented in Table 2 show that washed PEF, cotton, and rayon do not remove any Mg^{++} from solution while PAR binds all detectable Mg^{++} out of BHI.

Table 2. Removal of magnesium ion from BHI by different fiber types.

Extraction fiber	ug Mg^{++}/ml BHI
None	10
PEF (washed)	10
PAR	ND*
Cotton	12
Rayon	10

* ND = non-detectable at less than 2 ppm

Optimization of TSST-1 production in the presence of Fiber.

Influence of fiber concentration on TSST-1 production. In the following experiments the concentration of TSST-1 was

determined with a competitive EIA. In order to develop a standard fiber to medium ratio for evaluating TSST-1 production in the presence of fiber, S. aureus strain MN-8 was grown in BHI containing different concentrations of washed PEF and PAR fibers ranging from 1-10 % (w:v). As shown in Table 3, the concentration of fiber has a pronounced effect on TSST-1 production. As the concentration of the fiber increases the amount of TSST-1 produced per ml increases with maximum toxin levels occurring at 10 % fiber. Higher concentrations than 10 % were not tested because it was difficult to extract sufficient liquid from the fiber for testing even at 10 %. When TSST-1 production is normalized to 10^9 cfu the toxin production per organism appears to level off at 5 % for both fibers. Consequently the higher levels of TSST-1 at a concentration of 10 % fiber are from higher numbers of bacteria and not more toxin secreted per organism.

Table 3. Effect of fiber concentration on TSST-1 production.

Fiber	% Fiber (w:v)	TSST-1 ug/ml	TSST-1 ug/10^9 cfu
None-BHI control	-	0.2	0.1
PEF	1	0.8	0.3
	2.5	1.1	0.7
	5	3.5	1.2
	10	5.4	1.2
PAR	1	0.4	0.1
	2.5	1.1	0.4
	5	1.8	1.1
	10	3.0	0.8

Effect of surface to volume ratio on TSST-1 production. In a preliminary experiment it was found that toxin production was inhibited if the surface to volume ratio was low for cultures grown in the presence of PEF. When strain MN-8 was grown in 17 x 150 mm culture tubes with 10% fiber in BHI for a total of 10 ml, toxin production in the presence of PEF was dramatically decreased to below that of cultures with cotton or PAR (Table 4).

Table 4. TSST-1 production with low surface to volume ratio of culture medium.

Fiber	TSST-1 ug/ml	TSST-1 ug/10^9cfu
None-BHI control	0.1	<0.1
PEF	0.2	0.1
PAR	3.5	1.6
Cotton	1.6	0.2

When the test organism was grown in different volumes of BHI with the same concentration of fiber, it was found that changing the surface to volume ratio had opposite effects on PAR and PEF. Figure 22A shows the effect of different volumes on TSST-1 production per ml. An increase in volume and corresponding decrease in surface area had little or no effect on toxin production on the control grown in BHI only or BHI with added cotton. The PAR culture had an increase in toxin concentration per ml up to a volume of 2.5 ml and then toxin production remained stable. In contrast, the PEF culture had highest levels of toxin at a volume of 1 ml and decreased by half as the volume was increased to 5 ml. The difference from 5 to 10 ml did not appear to increase this effect. When the toxin production was corrected to

10^9 cfu (Figure 22B), volume had no effect on toxin production per unit organism in the control, cotton and PEF cultures but increased as volume was increased for the PAR fiber culture.

Aerated versus stationary culture. Cultures were incubated under stationary and aerated conditions. There was no significant difference in toxin production for cultures grown in the presence of PAR, cotton, CMC and controls without fiber whether aerated or not. PEF cultures produced almost twice the toxin per ml under aerated conditions in comparison to statically grown cultures (Table 5).

Table 5. Comparison of TSST-1 production in aerated versus stationary culture.

Fiber	ug TSST-1/ml Aerated	Stationary
None-BHI	0.1	0.1
PEF	7.0	3.7
PAR	3.1	2.7
Cotton	0.9	1.0

Figure 22. The effect of culture medium volume on TSST-1 production. Different volumes were compared maintaining the same percent fiber. Medium was inoculated with S. aureus strain MN-8. After 24 hours, culture supernatants were tested for TSST-1 by competitive ELISA.

Fiqure 22a

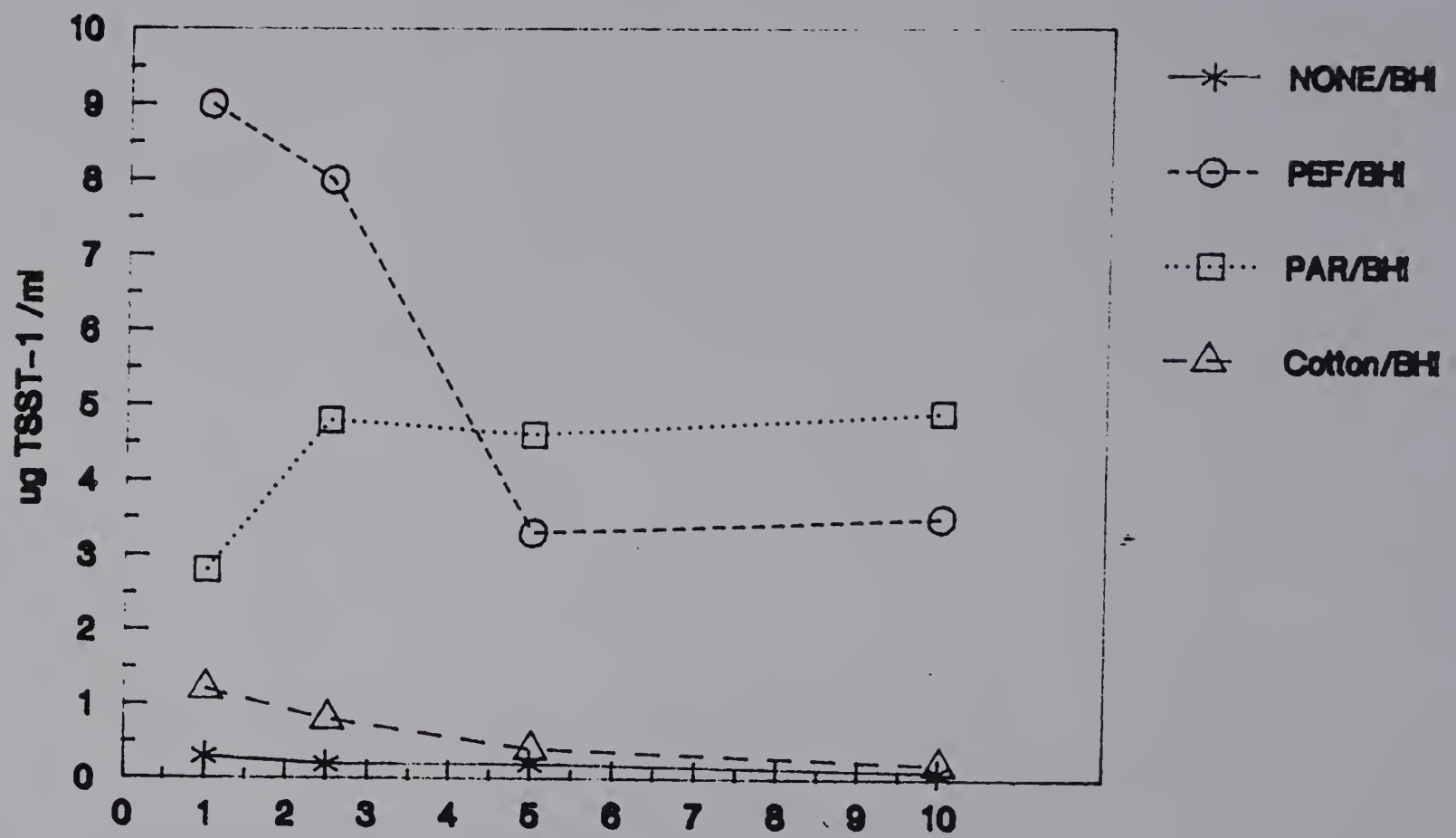
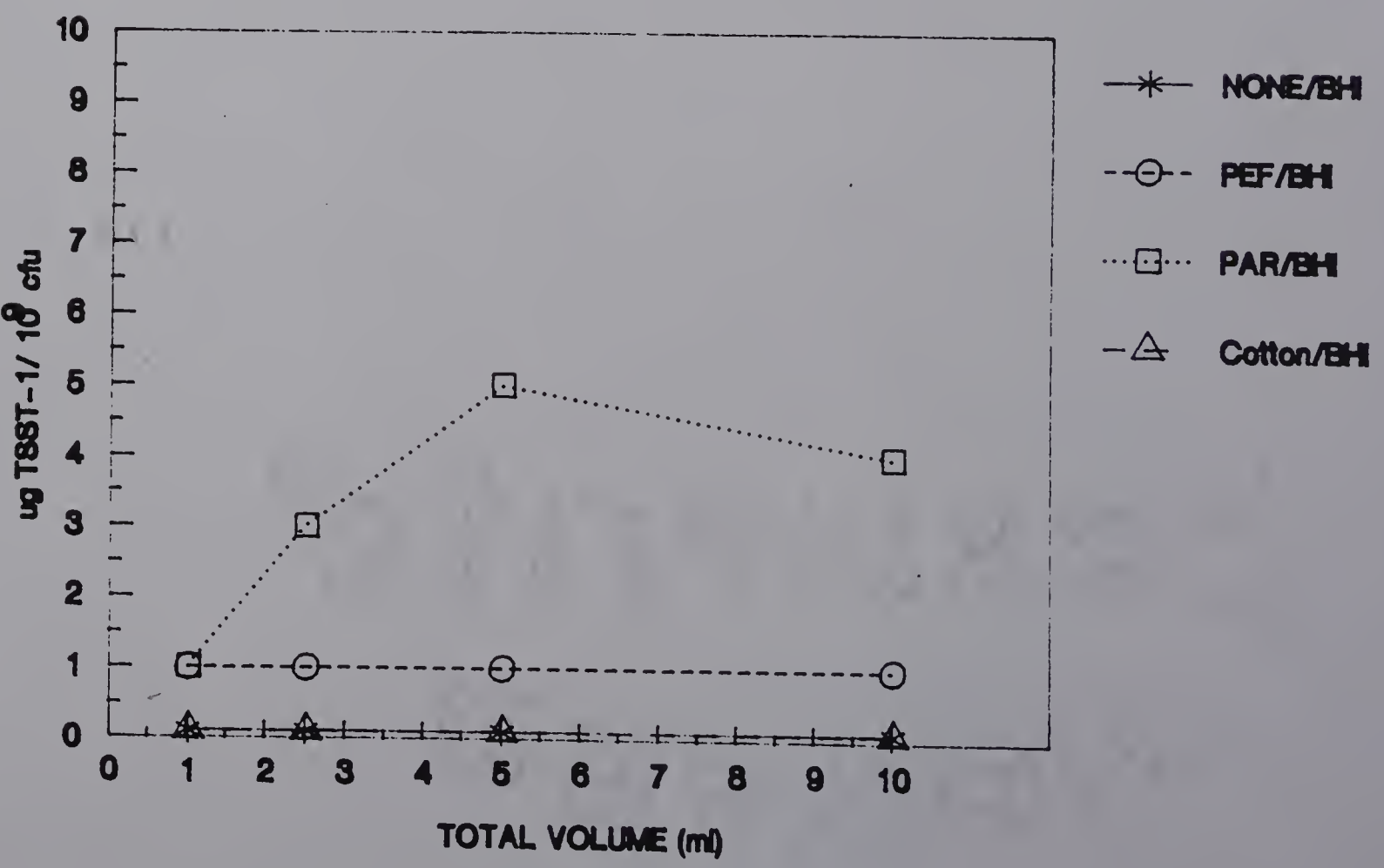


Figure 22b



Influence of inoculum procedure on TSST-1 production.

Different amounts of inoculum were compared for influence on TSST-1 production. An initial cell concentration of from 1 x 10^6 to 1 x 10^8 had little effect on TSST-1 produced per ml or per 10^9 cfu (Table 6).

Table 6. Influence of different cell numbers for initial inoculum on TSST-1 production

Fiber	Initial cfu/ml	TSST-1 ug/ml	TSST-1 ug/10^9 cfu
None	1 x 10^6	0.3	0.1
	1 x 10^7	0.3	0.3
	1 x 10^8	0.5	0.2
PEF	1 x 10^6	5.4	1.0
	1 x 10^7	6.5	1.1
	1 x 10^8	6.4	1.2
PAR	1 x 10^6	1.7	0.6
	1 x 10^7	2.1	0.8
	1 x 10^8	2.4	0.7

Two additional inoculation protocols using high and low numbers of rapidly growing cells were tested for effect on TSST-1 production. For both procedures, six hour cultures were used. In the first a 0.4 ml aliquot was added to 40 ml of BHI and the distributed into flasks containing fiber. The second consisted of centrifuging 40 ml of culture and resuspending in 40 ml of fresh BHI, followed by

distribution into flasks. The two procedures had minimal effect on the control without fiber and the culture containing cotton (Table 7). With PEF present in the medium twice as much TSST-1 was produced per ml using the second procedure in comparison to the first and there was no change in the amount produced per 10^9 cfu. In the PAR culture the first procedure enhanced toxin production about three fold over the second. Toxin production per cell unit was decreased by 10 fold in the second procedure in comparison to the first.

Table 7. Comparison of two inoculation procedures on TSST-1 production

Fiber	# 1* TSST-1 ug/ml	# 1* TSST-1 ug/10^9 cfu	# 2** TSST-1 ug/ml	# 2** TSST-1 ug/10^9 cfu
None-BHI	0.9	0.1	0.4	<0.1
PEF	1.7	0.23	3.2	0.3
PAR	4.2	1.2	1.0	0.2
Cotton	0.5	<0.1	0.5	<0.1

Preparation of inoculum: four colonies from a streak plate were inoculated into 50 ml of BHI and incubated for 6 hr while shaking.
*Procedure 1. 0.4 ml of the above was inoculated into 40 ml BHI and distributed into flasks containing fiber (5 ml/flask)
**Procedure 2. 35 ml of 6 hr culture was centrifuged and the pellet resuspended in fresh BHI and distributed into flasks.

Control of TSST-1 production by magnesium. Since it was established that PAR but not washed PEF could bind magnesium ions from BHI, growth and toxin production of S. aureus MN-8 was studied in fiber extracted medium with and without added Mg^{++}. As presented in Table 8, it was found the medium extracted with washed PEF had minimal effect on growth or TSST-1 production as compared to a non-fiber extracted control. Addition of magnesium from 10 to 100 ppm did appear to enhance growth slightly. In contrast, the PAR extracted medium did not support growth without addition of magnesium with the cfu per ml reaching only 4×10^5 in comparison to the control culture at 2.5×10^9 cfu/ml. Addition of Mg^{++} at concentrations of 10 and 25 ppm enhanced both growth and toxin production, with the culture reaching 2.3×10^9 cfu/ml at 25 ppm magnesium. Toxin production was also enhanced at levels of 10 and 25 ppm but decreased at higher concentrations both per ml and per 10^9 cfu.

Table 8. Growth and TSST-1 production by S. aureus MN-8 in fiber extracted medium with added magnesium.

Extraction Fiber	added Mg^{++} ppm	cfu per ml	TSST-1 ug/ml	TSST-1 ug/10^9 cfu	ppmMg^{++} AA*
None-BHI	0	2.5×10^9	0.7	0.3	13
PEF	0	2.1×10^9	0.1	<0.1	12
	10	6.7×10^9	0.1	<0.1	-
	25	2.4×10^9	0.1	<0.1	-
	50	5.8×10^9	0.1	<0.1	-
	100	3.1×10^9	0.1	<0.1	-
PAR	0	4.0×10^5	<0.1	<0.1	0.9
	10	1.5×10^9	1.2	0.8	-
	25	2.3×10^9	1.1	0.5	-
	50	2.3×10^9	0.9	0.4	-
	100	3.6×10^9	0.8	0.2	-

*AA, atomic absorption spectrophotometry

Different concentrations of magnesium were added to BHI containing PEF and PAR (Table 9) and inoculated using a standard inoculation procedure (50 ul of a 6 hr culture inoculated into 5 ml of BHI containing 10 % fiber). Addition of magnesium to the control cultures without fiber had no significant effect on toxin production. In cultures grown in the presence of PEF, TSST-1 levels at 0 ppm added Mg^{++} were at 9.2 ug/ml and they decreased to 5.6 ug/ml at

100 ppm added magnesium. The TSST-1 levels when corrected to ug per 10^9 cfu were 9.2 ug at 0 ppm added Mg^{++} and decreased to 1.5 ug at 100 ppm. The PAR also decreased TSST-1 levels almost two fold as magnesium levels were increased from 0 to 100 ppm for both ug/ml and ug per 10^9 cfu.

Table 9. Effect of added magnesium in the presence of fiber on TSST-1 production

Fiber	Added Mg^{++} ppm	TSST-1 ug/ml	TSST-1 ug/10^9 cfu
None-BHI	0	0.1	0.1
	10	0.1	0.1
	50	0.2	0.1
	100	0.1	<0.1
PEF	0	9.2	9.2
	10	7.8	5.2
	50	6.6	2.3
	100	5.6	1.5
PAR	0	2.5	2.1
	10	1.1	1.2
	50	1.1	0.6
	100	1.4	1.3

Fiber/BHI medium was inoculated with 50 ul of a 6 hr culture.

In a second similar experiment, cultures of S. aureus were concentrated by centrifugation, resuspended in fresh BHI at a concentration of 10^{10} cfu/ml and then distributed into flasks containing fiber. Interestingly, the cultures

in the presence of PEF demonstrated the same trend as in the previous experiment in that added magnesium from 0 to 100 ppm decreased the amount of toxin produced by about half both per ml and 10^9 cfu (Table 10). The opposite was observed for the PAR grown cultures, with 2.5 ug/ml TSST-1 produced at 0 ppm Mg^{++} and increasing up to 10.6 ug toxin per ml with 100 ppm magnesium. The toxin produced per ml followed a similar pattern.

Table 10. Effect of added magnesium on TSST-1 production in the presence of fiber and a concentrated cell inoculum

Fiber	Added Mg^{++} ppm	TSST-1 ug/ml	TSST-1 ug/10^9 cfu
None-BHI	0	0.2	<0.1
	10	0.2	<0.1
	50	0.1	<0.1
	100	0.1	<0.1
PEF	0	4.9	0.4
	10	5.8	0.4
	50	2.5	0.2
	100	2.8	0.2
PAR	0	2.5	0.3
	10	8.0	0.8
	50	7.7	0.8
	100	10.6	1.1

Response of Guinea Pigs to Intravaginal inoculation with TSS associated strains of S. aureus.

When the relationship of TSS to menstruation was first established, the prevalence of tampons composed of certain high absorbency fibers became apparent on completion of epidemiological studies (Shands et al.,1980, Davis et al.,1980). One of the brands of tampons that was frequently associated with the occurrence of was Rely, which contained CMC and PEF, two highly absorbent fibers. An early study conducted by Tierno et al (1983) indicated that CMC might be involved in the pathogenesis of TSS. With this in mind, an experiment was conducted to assess the effect of CMC in combination with TSS associated strains of S. aureus on guinea pigs. This species had not been previously tested for sensitivity to TSS associated organisms.

Five female guinea pigs were initially screened for the presence of S. aureus by culturing intravaginal specimens. S. aureus was not cultured from any of the vaginal specimens obtained prior to inoculation. Blood samples were collected from each guinea pig before the animals were inoculated intravaginally with a small piece of Weck-cell sponge (Edward Weck and Co. Inc. NY) and a pellet of CMC from a Rely tampon. Twenty ul of a 24 hr culture containing approximately 2×10^7 cfu was then inoculated into the vagina of each animal. The TSS associated strains were MN-

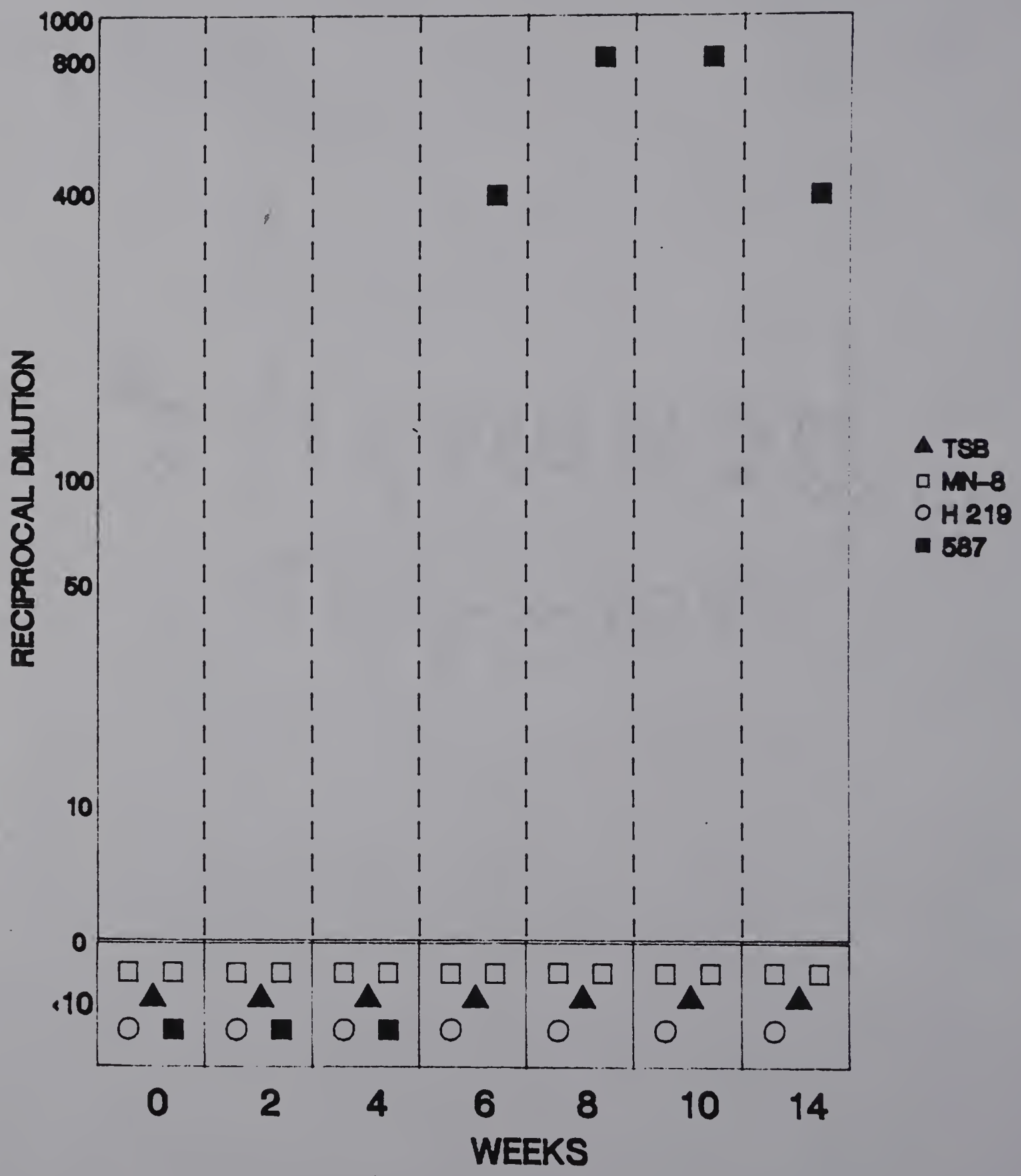
8 and 587 and the control strain was H219, a non-TSS associated vaginal isolate. One guinea pig received the fibers and uninoculated medium as a control. The inoculation procedure was repeated at 2, 4, 6, 8, 10, and 14 weeks. In addition, the animals' temperature was monitored daily from the day before to one week after the first four inoculations.

The guinea pigs did not have any observable physiological changes that could be attributable to the intravaginal inoculations. There was no increase in the animals' body temperatures.

A histogram of the serum titers of all the animals is shown in Figure 23. Serum samples were serially diluted and assayed against ^{125}I labelled TSST-1 using fixed cowan 1 cells as an immunoprecipitant. The reciprocal of the last dilution with a signal greater than control sera was the titer. Of the 5 animals tested, only the one inoculated with S. aureus 587 developed a specific IgG response to TSST-1 as determined by RIA. Serum antibody to TSST-1 was not detectable until 6 weeks after the initial inoculation. If the animal had responded to the first inoculation, serum reactivity to TSST-1 would have appeared in the samples taken earlier than 6 weeks. Using this protocol, it appears that repeated inoculation was necessary for an immune response to TSST-1 to occur. This inoculation procedure in combination with the fibers and

Figure 23: Serum antibody response to intravaginal inoculation with Weck-cell sponge and CMC and S. aureus. Guinea pigs were implanted with a Weck-cell sponge and a CMC pellet from a Rely tampon and inoculated with 2×10^7 cfu intravaginally. TSS associated strains MN-8, 587, and the non-TSS associated strain H219 were used. One animal received uninoculated medium alone (TSB). Anti-TSST-1 serum titers were determined by RIA.

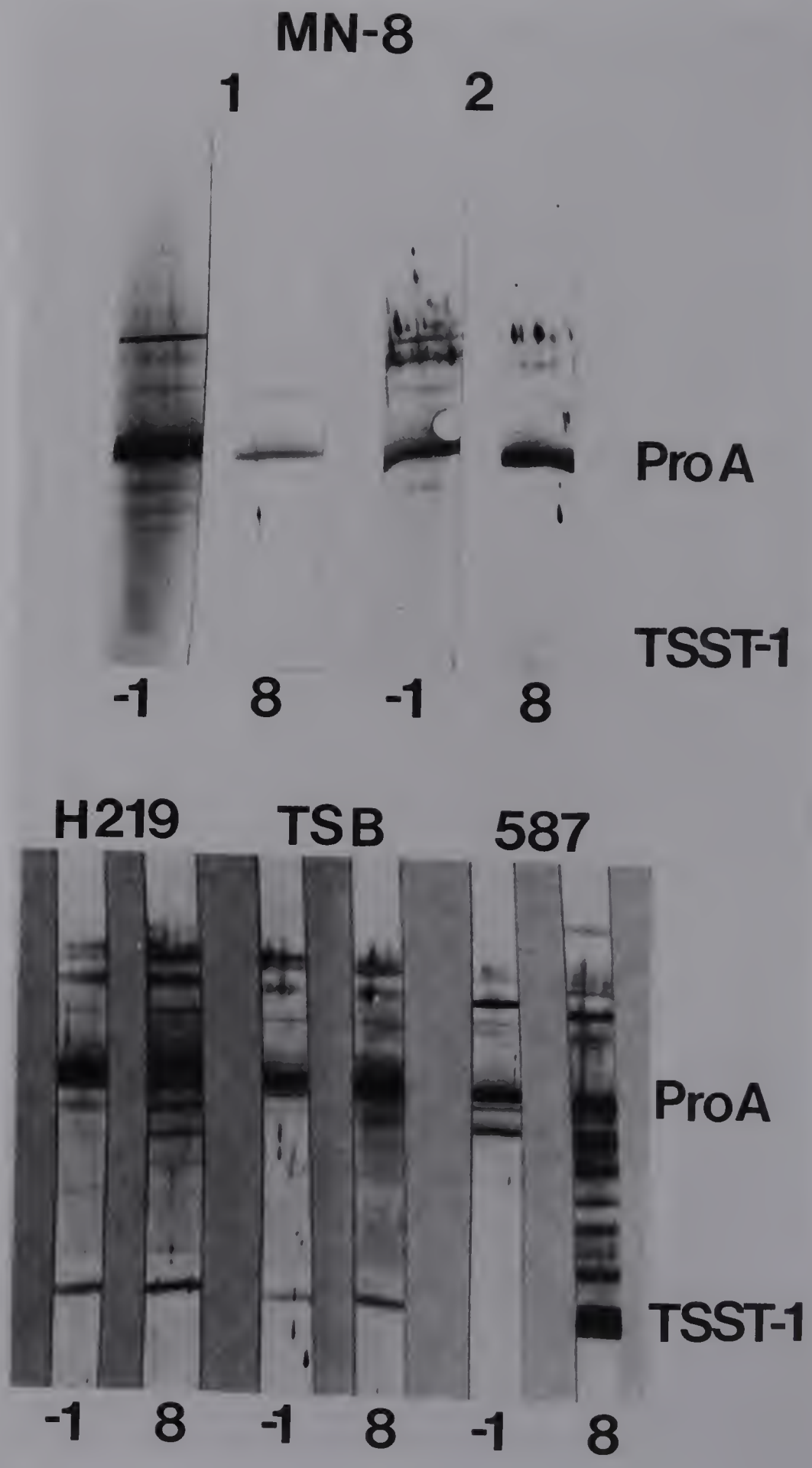
Figure 23


1000
800
400
100
50
10
0
<10
RECIPROCAL DILUTION
0
2
4
6
8
10
14
WEEKS
TSB
MN-8
H 219
587

organisms used, is not particularly conducive to either intravaginal production of TSST-1 or a corresponding antibody response.

Sera collected before and 12 weeks after the initial infection were also analyzed by immunoblotting. Ammonium sulfate concentrated culture supernatants from S. aureus MN-8 were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Figure 24 shows the reaction pattern of the different serum samples to nitrocellulose strips containing MN-8 proteins. Only the animal inoculated with strain 587 that had a positive serum response by RIA, has a distinct change in the reaction pattern against the extracellular proteins from strain MN-8. A strong reaction to the TSST-1 band is evident and several other bands in the molecular weight range of 18 to 40 kDa are also visible in the post-inoculation serum. Interestingly, the animals that were inoculated with MN-8, another TSS associated strain, had no change in the serum antibody reactivity to any of the MN-8 proteins. This indicates that under these conditions with the exception of the strain 587 inoculated animal, the guinea pigs are not only unreactive to TSST-1 but also are not producing a detectable response to any other strain MN-8 associated extracellular protein that was used for the blot analysis. In this light, it is noteworthy that the serum sample from the anti-TSST-1 antibody positive animal inoculated with

Figure 24. Immunoblot of guinea pig sera after intravaginal inoculation with fiber and S. aureus. Culture supernatants from S. aureus MN-8 (TSST-1 producing strain) were resolved by SDS-PAGE and transfered to nitrocellulose membranes. Guinea pig sera from 1 week before inoculation (-1) and 8 weeks post inoculation with S. aureus strains MN-8, H219, 587 or culture medium alone (TSB). Serum antibodies reacting with proteins on the nitrocellulose membranes were detected using a HRP-goat anti-guinea pig IgG conjugate.

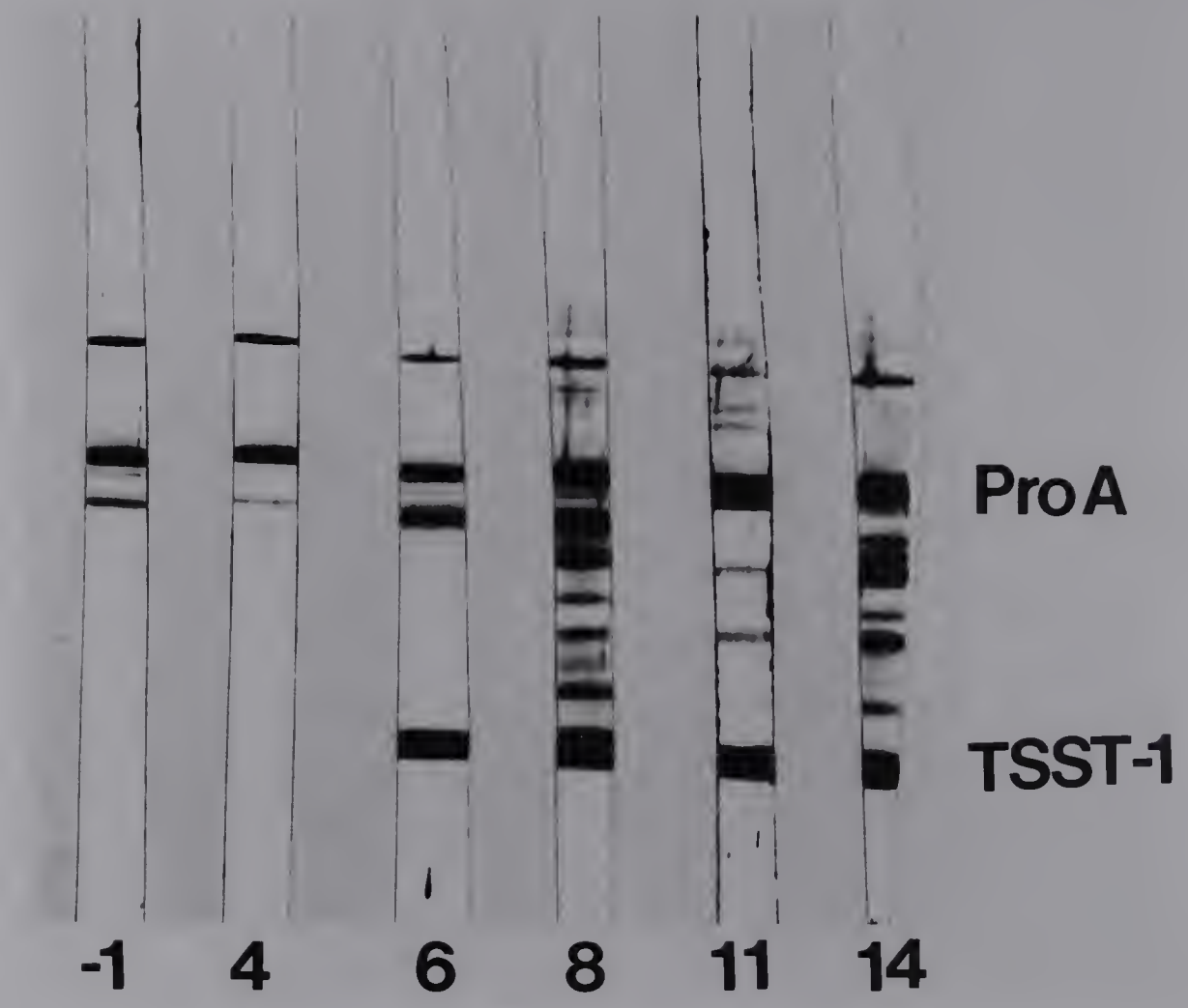

MN-8
1
2
Pro A
TSST-1
-1
8
-1
8
H219
TSB
587
ProA
TSST-1
-1
8
-1
8
-1
8

strain 587 is reactive to many of the MN-8 proteins in addition to TSST-1. The temporal antibody response of this animal is shown in Figure 25. Reactivity to TSST-1 is first seen in a serum sample from 6 weeks after the first inoculation. The band at about 40 kDa that was consistently reactive to all the sera tested is protein A. This protein binds non-specifically to the Fc region of IgG and is produced by many strains of S. aureus. Several other bands between the 22 kDa TSST-1 band and the 40 kDa protein A band were visible beginning at week 8. The banding pattern for this region remains constant for weeks 8-14.

Subsequent to the completion of this experiment it was found that CMC and the Weck-cel sponge, the fibers used for intravaginal implantation, did not enhance the in vitro production of TSST-1 to the extent that certain other fibers did. In addition, TSB was not found to be as conducive to TSST-1 production as BHI. The influence of various fibers in BHI on the concentration of TSST-1 produced per ml of culture by S. aureus MN-8 is shown in Table 11. A quantitative competitive inhibition ELISA was used to measure the concentration of TSST-1. If TSST-1 was present in the sample, binding of the conjugate (TSST-1-alkaline phosphatase) to the rabbit anti-TSST-1 antibody coating the wells was inhibited producing a quantifiable decrease in signal. Polyester foam (PEF) from the Rely

Figure 25. Temporal antibody response to intravaginal inoculation with S. aureus strain 587. Culture supernatants from S. aureus MN-8 were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blood was collected from guinea pigs at one week before and 4, 6, 8, 11 and 14 weeks post inoculation. Sera from the animal inoculated with a Weck-cell sponge and CMC containing S. aureus strain 587 was incubated with the nitrocellulose membranes. Antibodies were detected with HRP-goat anti-guinea pig IgG conjugate.

587



tampon was shown to induce 5.3 ug of TSST-1 per ml which was the most of any of the fibers tested. This was 10 times the amount of toxin produced in control cultures without fiber. Polyacrylate rayon (PAR) induced 2.8 ug/ml of TSST-1 or approximately 5 times the control culture concentration. PAR was found in several brands of tampons prior to its removal from the market by all manufacturers in 1985. In contrast, CMC and the Weck-cel sponge supported the least amount of toxin production among all the fibers. Supernatants from cultures grown in the presence of these two fibers contained less TSST-1 than controls, 0.1 versus 0.5 ug per ml, respectively.

Table 11. TSST-1 production in the presence of different tampon fibers by strain MN 8.

Medium	fiber	TSST-1 ug/ml
TSB	none	0.2
BHI	none	0.5
BHI	Rely	
	outer wrap	1.3
	CMC	0.1
	PEF	5.3
BHI	PAR	2.8
BHI	Rayon	0.8
BHI	Cotton	0.7
BHI	Weck-Cell sponge	0.1

Because of the different effects that each type of fiber had on TSST-1 production and the information reported by Schlievert (1983) that strain MN-8 produces about 10 times more TSST-1 than strain 587 does under the same conditions, it was decided to attempt a second experiment with several changes in the protocol that was used in the first guinea pig experiment. These changes reflected the in vitro data and the relationship of specific fibers to production of TSST-1 (Table 11). It seemed possible that the conditions that were found to enhance TSST-1 production in vitro might induce symptoms of TSS in a guinea pig model. The response

of guinea pigs to intravaginal inoculation was again tested under conditions that were know to maximize toxin production. This included the use of PEF, a minimal amount of medium (BHI) to moisten the fiber and strain MN-8 which produces more TSST-1 than most other TSS associated strains of S. aureus.

Twelve female guinea pigs between 4-6 months of age were prescreened for vaginal colonization of S. aureus by collecting intravaginal specimens with cotton tipped swabs. No S. aureus was found in any of the cultured swabs. Since one of the key symptoms of TSS is fever, the animals were monitored daily to determine their baseline temperatures beginning 1 month prior to inoculation and continued for one week after the inoculation period.

Fibers used in this study consisted of PEF from Rely super absorbent tampons. PEF pieces, about 0.5 cm^2 in size, were separated from the CMC pellets and the PEF was covered with Rely polyester overwrap and tied at the bottom with withdrawal cord. Cotton from original regular tampons (Tambrands) was also covered with the Rely overwrap and tied with the same cord. Immediately before inoculation of the guinea pigs, each of the wrapped fiber pieces were dipped into BHI and then squeezed out as much as possible. One of these pieces was inserted into the vagina of each anaesthetized animal. The animals were then inoculated intravaginally with 20 ul of S. aureus strain MN-8 or 6538p

($2x10^7$ cfu).

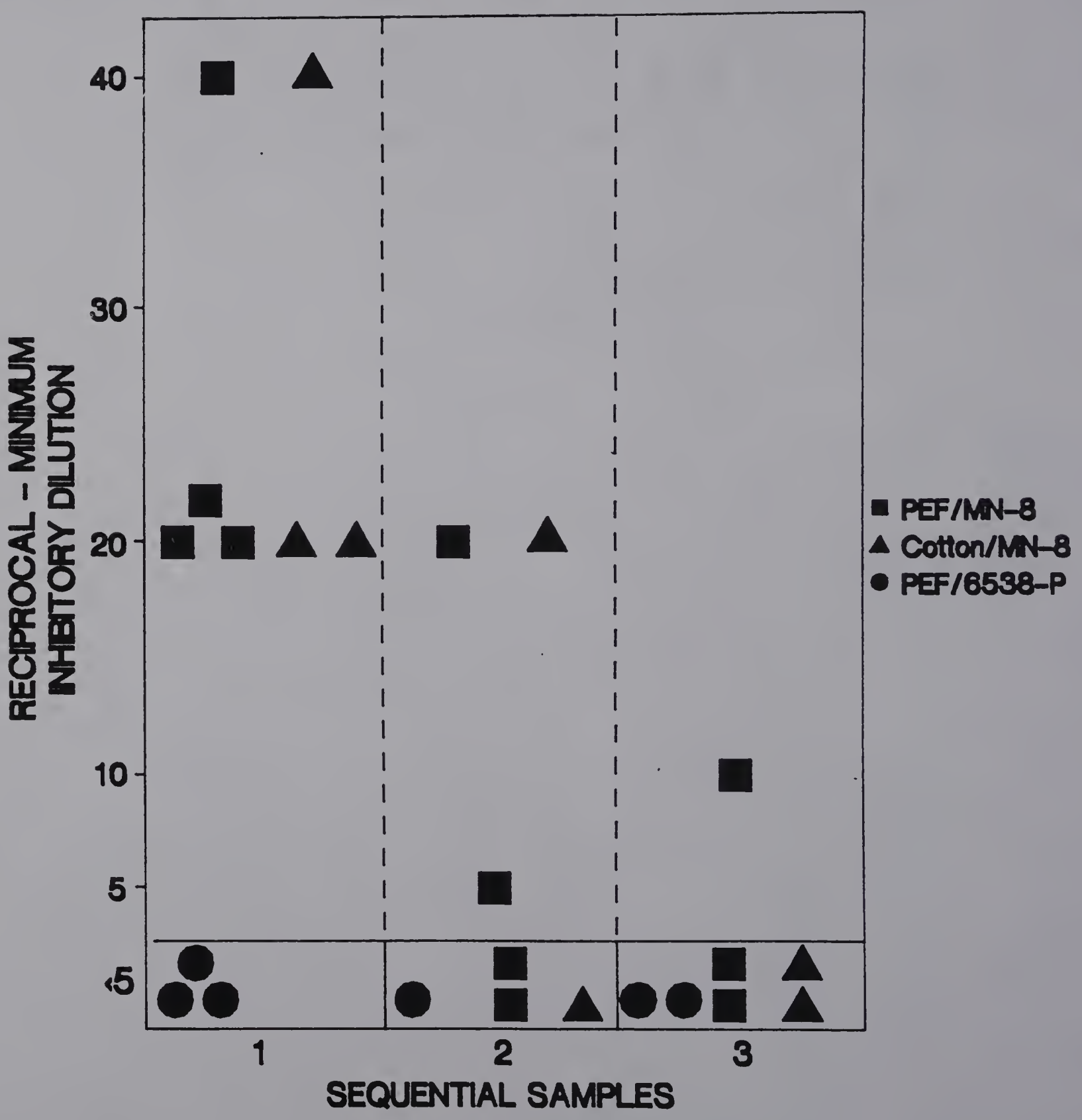
After 48 hr the fiber pieces were removed and replaced with fresh ones and the animals were reinoculated. This inoculation procedure was repeated a total of 3 times over 6 days. Each fiber piece that was recovered was extracted by triturating in sterile PBS. An aliquot of this was cultured on blood agar plates to confirm the presence of the inoculated organism. The rest of each sample was centrifuged and the supernatant frozen for later analysis for TSST-1 concentration.

In all of the post inoculation fiber samples that were recovered or alternately swabs that were used for sampling if the fiber sample could not be recovered, the inoculated organisms (S. aureus MN-8 or 6538-p) were found as the predominate microflora. The normal microflora seen in the pre-inoculation specimens was still present in the post-inoculation samples but appeared to have been outgrown by the staphlococci under these conditions.

Fiber extracts from intravaginal incubation were tested for the relative concentration of TSST-1 by a competitive inhibition RIA. If TSST-1 was present in the sample the amount of binding of rabbit anti-TSST-1 to labelled toxin was decreased. In contrast to the in vitro data (Table 11) where PEF stimulated far more TSST-1 than did cotton, comparable amounts of TSST-1 were found in the in vivo samples for both fibers (Figure 26). It was surprising

Figure 26: TSST-1 levels in fiber extracts after intravaginal incubation with S. aureus. Guinea pigs were inoculated with polyester foam and TSST-1 producing strain MN-8 (PEF/MN-8), cotton and TSST-1 producing strain MN-8 (Cotton/MN-8), or polyester foam and a non-TSST-1 producing strain 6538-p (PEF/6538-p). Fibers were extracted and the TSST-1 levels were determined by a competitive inhibition RIA.

Figure 26


RECIPROCAL - MINIMUM INHIBITORY DILUTION
40
30
20
10
5
<5
1
2
3
SEQUENTIAL SAMPLES
PEF/MN-8
Cotton/MN-8
PEF/6538-P

that guinea pigs implanted with cotton and inoculated with MN-8 had levels of TSST-1 comparable to those that received the PEF/MN-8 combination. The minimum inhibitory dilutions for all the cotton and PEF samples retrieved were either 1/20 or 1/40. As expected, there was no detectable TSST-1 produced in the samples from the guinea pigs implanted with PEF and the non-TSST-1 producing strain, S. aureus 6538-p. It was also found that for each consecutive sampling, the proportion of TSST-1 positive samples decreased (Table 12). The first time samples were recovered, 7 out of 7 of the fiber samples that were inoculated with MN-8 contained detectable levels of TSST-1 while on the second sampling the proportion of positive samples decreased to 2 out of 6 fiber pieces recovered. At the third and last sampling, only 1 out of 5 fiber extracts contained TSST-1.

Table 12. Detection of TSST-1 in sequential samples of guinea pig vaginal fiber implants.

		TSST-1 positive/samples recovered Sample #		
Strain	Fiber	1	2	3
6538 p	PEF	0/3	0/1	0/2
MN 8	PEF	4/4	1/4	1/3
MN 8	Cotton	3/3	1/2	0/2

Each animal was monitored daily for basal body

temperature beginning from one month before to one week after the first inoculation. Only the control group inoculated with strain 6538-p had a statistically significant increase in temperature corresponding to the inoculation period. Table 13 shows the average temperatures for each group for the week during inoculation and one week before.

Table 13. Effect of inoculation on guinea pig temperatures.

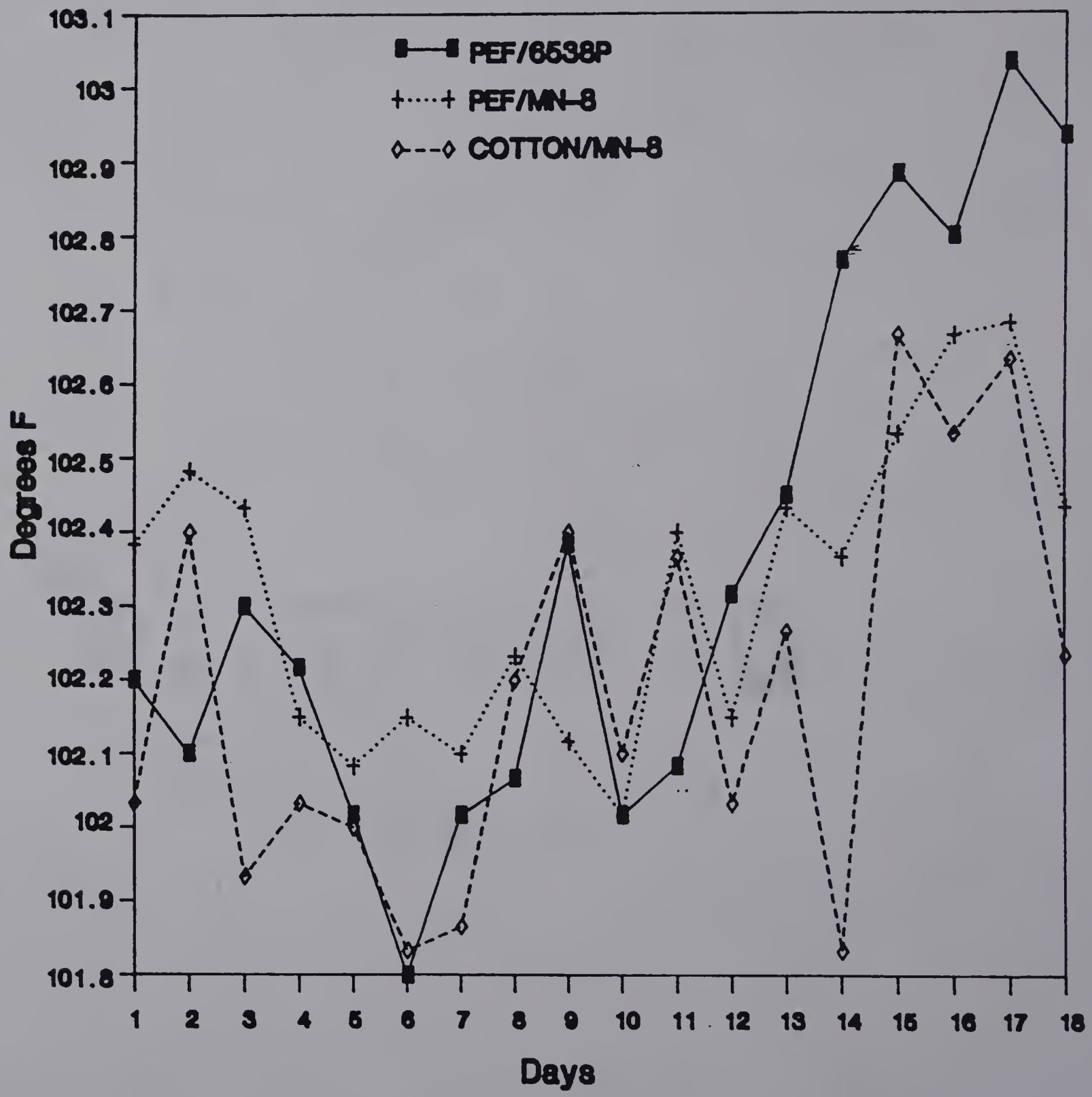
	Average temperatures Group		
	1 PEF/6538 p	2 PEF/MN 8	3 Cotton/MN 8
Before inoculation	102.2	102.4	102.1
During inoculation	102.9	102.5	102.4

Even though there was not a significant change in temperatures of the PEF/MN-8 and Cotton/MN-8 groups there is a distinct trend towards higher readings of both these groups during the inoculation period as shown in Figure 27. This is indicative that these animals are responding to the inoculation procedure.

Serum and eye secretions were collected from each animal prior to the inoculation regime and then at 1,2,3,4,5,7,and 11 weeks following the inoculation period. The tears were

Figure 27. Basal body temperatures of guinea pigs inoculated with S. aureus. Guinea pig temperatures were monitored for one month before and one week after intravaginal inoculation with tampon fibers and S. aureus.

Figure 27


PEF/6538P
PEF/MN-8
COTTON/MN-8
Degrees F
103.1
103
102.9
102.8
102.7
102.6
102.5
102.4
102.3
102.2
102.1
102
101.9
101.8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Days

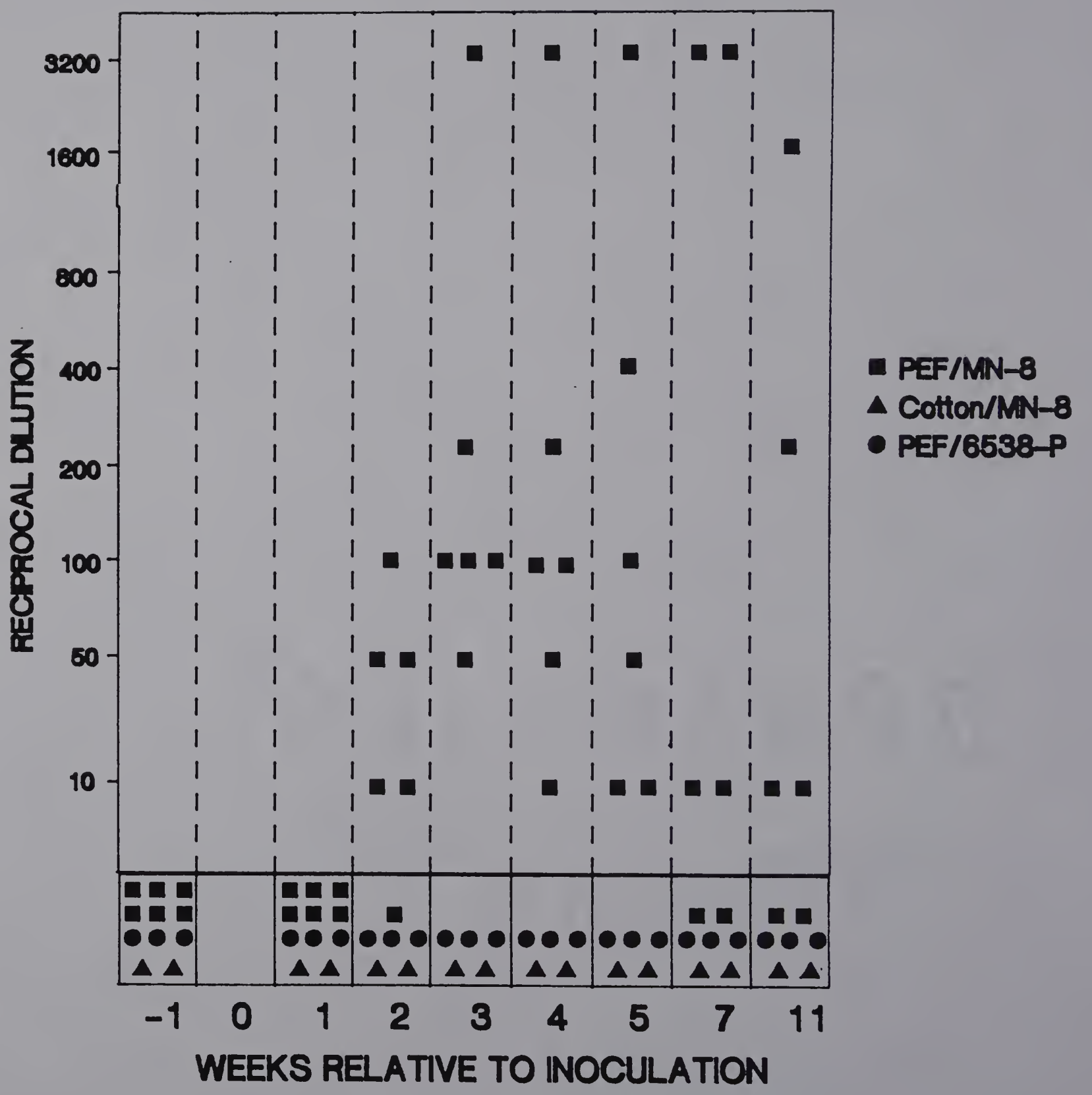
analyzed for a specific IgA response to TSST-1 by competitive inhibition RIA, a specific IgA capture ELISA and by immunoblotting using goat anti-guinea pig IgA prepared against colostrum. There was no detectable immune response to TSST-1 in the tears.

Sera was analyzed for a TSST-1 specific IgG response using a liquid phase RIA and fixed cowan 1 cells as an IgG specific immunoprecipitate. Even though there were comparable amounts of TSST-1 found in the vaginal samples of the animals implanted with cotton or PEF, only the animals implanted with PEF and inoculated with MN-8 elicited a humoral response to TSST-1 (Figure 28). Titers of anti-TSST-1 antibody were present in 5 out of 6 animals by the second week following the inoculation period. By the third week, all the animals from the PEF/strain MN-8 group showed titers ranging from 1/50 to 1/3200. At dilutions of 1/10 the control animals implanted with PEF and strain 6538-p and the cotton implanted animals had no detectable serum antibody response throughout the period of sampling. One animal that received the cotton/MN-8 combination died the week following the inoculation period due to unrelated causes and was not included in this figure.

Preinoculation and 4 week post inoculation sera samples from three animals in each treatment group (2 in the cotton/MN-8 group) were analyzed by immunoblot to determine

Figure 28. Serum IgG response to intravaginal inoculation with tampon fibers and S. aureus. Guinea pigs were inoculated with polyester foam and TSST-1 producing strain MN-8 (PEF/MN-8), cotton and TSST-1 producing strain MN-8 (Cotton/MN-8), or polyester foam and non-TSST-1 producing strain 6538-p (PEF/6538-P). Animals were bled one week before inoculation (-1) and on weeks 1, 2, 3, 4, 5, 7 and 11 after inoculation. Serum titers were determined by RIA.

Figure 28


3200
1600
800
400
200
100
50
10
RECIPROCAL DILUTION
-1
0
1
2
3
4
5
7
11
WEEKS RELATIVE TO INOCULATION
PEF/MN-8
Cotton/MN-8
PEF/6538-P

immunoreactivity against TSST-1 and other extracellular proteins from S. aureus MN-8. Extracellular proteins from strain MN-8, concentrated by ammonium sulfate precipitation (0-90% saturation) and resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes. All sera were diluted to 1/100 prior to probing the membranes. Reaction patterns of sera from the animals in the control group (PEF/strain 6538-p) show very little change in reactivity against any of the MN--8 proteins (Figure 29). Animal #1 did develop antibodies against two proteins at molecular weights of about 30 and 35 kDa. These appear below the strongly staining 40 kDa band. There is no change in the area of the blots probed with sera from these animals that corresponds to the TSST-1 band.

In the second group of animals (PEF/strain MN-8) which all had a titrable TSST-1 specific antibody response as determined by RIA (Figure 30), there is clearly reactivity in all the post inoculation sera samples to TSST-1. No reactivity to TSST-1 is present in the prebleed sera. In addition to the reaction with TSST-1 there is also development of a band below the protein A band which was visible in the sera of the animals inoculated with strain 6538-p.

The temporal immunoreactivity patterns seen in one animal from the PEF/strain-MN-8 group is shown in Figure 31. This

Figure 29. Immunoblot analysis of guinea pig sera after intravaginal inoculation with fiber and S. aureus. Animals were inoculated with polyester foam and strain 6538-p. Culture supernatants from TSST-1 producing strain MN-8 were resolved by SDS-PAGE and transferred to nitrocellulose. Sera were taken one week before and seven weeks after inoculation were detected with goat anti-guinea pig IgG HRP conjugate.

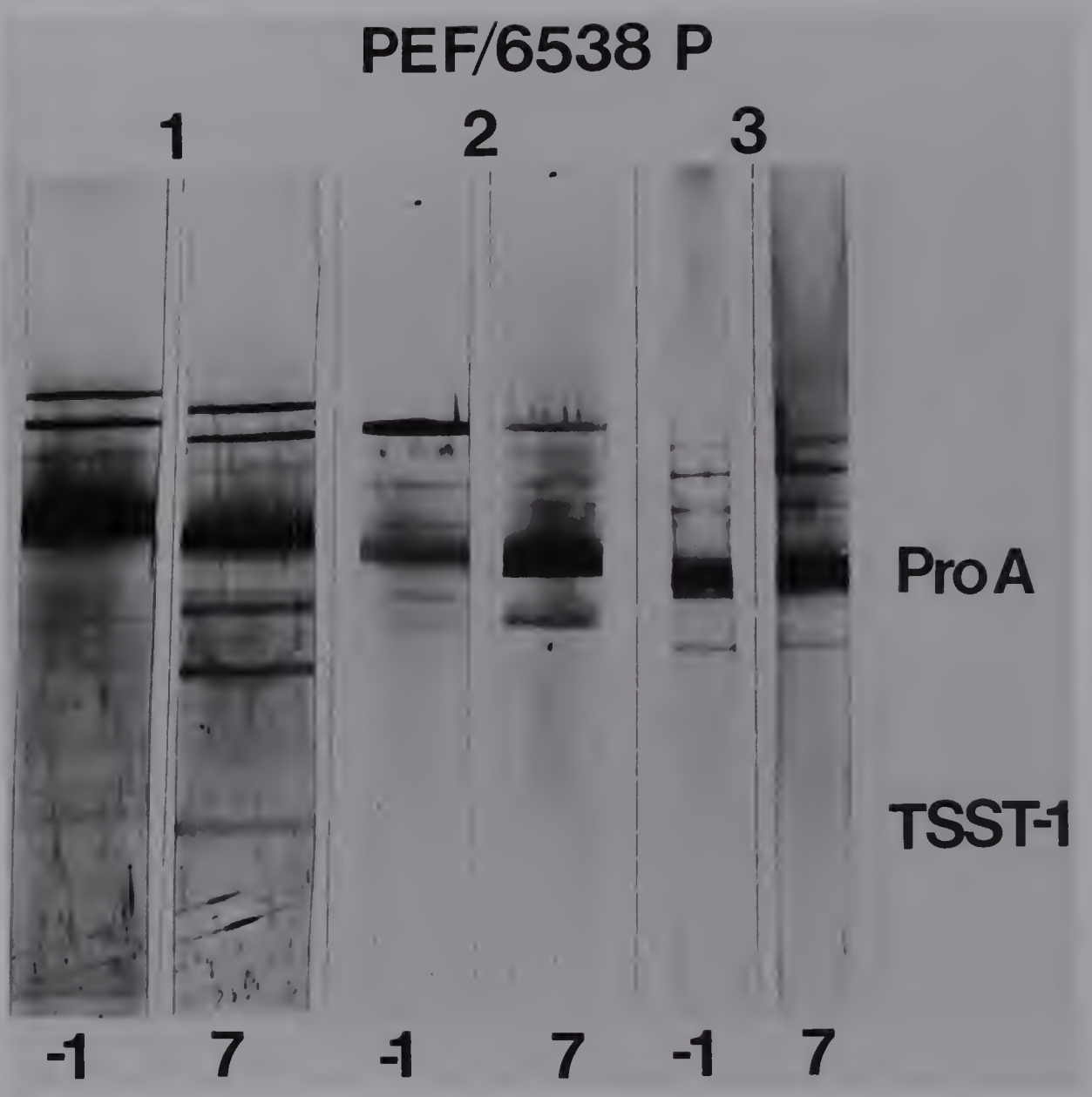
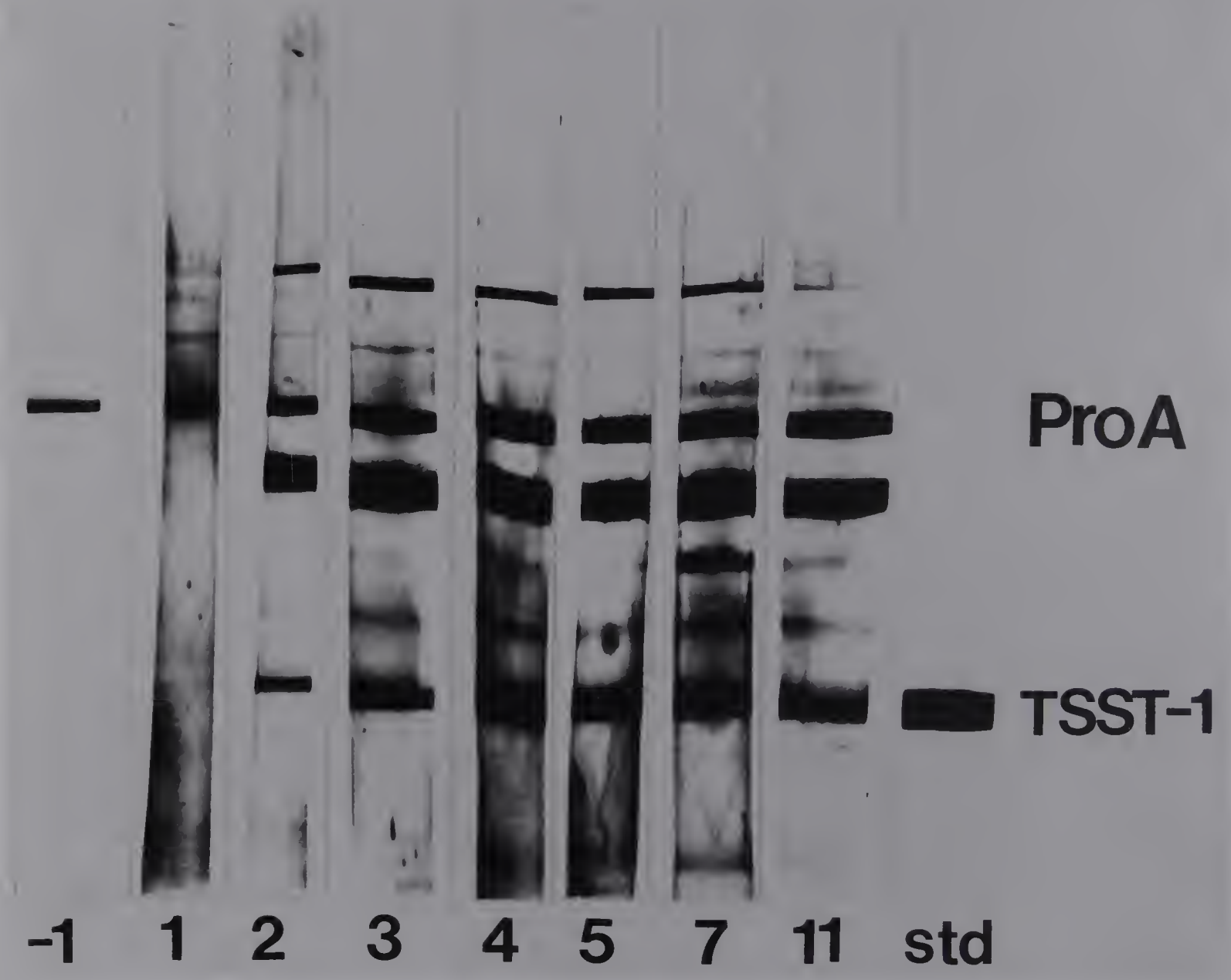

PEF/6538 P
1
2
3
Pro A
TSST-1
-1
7
-1
7
-1
7

Figure 30. Immunoblot analysis of guinea pig sera after intravaginal inoculation of polyester foam and strain MN-8. Culture supernatants from TSST-1 producing strain MN-8 were resolved by SDS-PAGE and transferred to nitrocellulose. Animals were inoculated with polyester foam and strain MN-8. Sera taken one week before and seven weeks after inoculation were assayed against the nitrocellulose membranes and detected with goat anti-guinea pig IgG HRP conjugate.


PEF/MN8
1
2
3
ProA
TSST-1
-1
7
-1
7
std
-1
7
std

Figure 31. Temporal antibody response to intravaginal inoculation with polyester foam and S. aureus MN-8. Culture supernatants from S. aureus MN-8 were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Serum was collected from guinea pigs one week before and 4, 6, 8, 11 and 14 weeks post inoculation. Sera were incubated with the nitrocellulose membranes and detected with goat anti-guinea pig IgG HRP conjugate.

PEF/MN 8



particular animal had titers from 1/100-1/3200, one of the highest titers of the group in several consecutive serum samples. This series of blots shows the rapid development of immunoreactivity against the 22 kDa TSST-1 band, the 35 kDA band, several other bands between protein A and TSST-1 and one in the 60 kDa region.

Immunoblot patterns from sera of the last group of animals (cotton/strain MN-8) are shown in Figure 32. These sera react with only the 40 kDa protein band and lack any reactivity with the TSST-1 band. The results of the immunoblots are consistent with the RIA results and show no development of anti-TSST-1 activity. The animals showed very little change in antibody reactivity in the post-inoculation sample as compared to the prebleed. These results indicate that PEF but not cotton causes a fiber specific enhancement of the serum antibody response to TSST-1 and other unidentified staphylococcal extracellular proteins.

Figure 32. Immunoblot analysis of guinea pig sera after intravaginal inoculation of cotton and strain MN-8. Culture supernatants from TSST-1 producing strain MN-8 were resolved by SDS-PAGE and transferred to nitrocellulose. Serum was taken one week before and seven weeks after inoculation and assayed against the nitrocellulose membranes. Antibodies reacting with the nitrocellulose bound proteins were detected with goat anti-guinea pig IgG HRP conjugate.

Figure 32. Immunoblot analysis of guinea pig sera after intravaginal inoculation of cotton and strain MN-8. Culture supernatants from TSST-1 producing strain MN-8 were resolved by SDS-PAGE and transferred to nitrocellulose. Serum was taken one week before and seven weeks after inoculation and assayed against the nitrocellulose membranes. Antibodies reacting with the nitrocellulose bound proteins were detected with goat anti-guinea pig IgG HRP conjugate.

C H A P T E R I V

DISCUSSION

Purification and characterization of toxic shock syndrome toxin-1. The initial effort in these studies was concentrated on identification, purification and characterization of TSST-1. As all subsequent work was based on having the ability to detect the toxin itself and monitor the production of TSST-1 specific antibodies, this first step was critical to the outcome of the project. Preliminary identification of a protein reactive with a standard preparation of rabbit anti-TSST-1 antiserum (FS-12, M.S. Bergdoll) provided a means to identify TSST-1 and evaluate and develop methods for purification of additional toxin.

The schemes used for purification of TSST-1 all resulted in an essentially homogeneous preparation. One of the problems encountered in isolation of the protein is that it is only present in small amounts (<5 ug/ml) in culture supernatants in comparison to other extracellular proteins or peptides from the medium preparation. In early efforts to purify the toxin, brain heart infusion dialysate was used as a culture medium. It was later found that most of the proteins present in commercially available BHI were of low molecular weight and as such, did not interfere with the purification protocols.

Some of the modifications in the purification schemes were made to improve the yield of TSST-1. The effluent after adsorption of the culture supernatant onto to a weak cation exchange resin (CM Sephadex) was found to still contain most of the TSST-1 that was present before adsorption. This resulted in trials with a stronger ion exchange resin, Rexyn 102. This resin adsorbed toxin to the extent that there was no detectable toxin in the supernatant. On the other hand, using Rexyn 102 did not yield as clean a toxin preparation as CM Sephadex. Additional purification with a combination of ammonium sulphate precipitation and molecular exclusion chromatography did result in a highly purified preparation of the toxin.

Although purification by affinity chromatography with goat anti-TSST-1 yielded a single band of protein with a minimum number of steps, it was difficult to purify sufficient toxin with this procedure. Only small amounts of toxin (<50 ug) could be purified at a time.

The toxin has been described as having a molecular weight ranging from 19.2 kDa (Reeves et al.,1986) up to 24 kDa (Igarashi et al.,1984). Reeves and coworkers found that the toxin had a molecular weight of 19.2 kDa using hydrodynamic methods which is close to the 20 kDa reported by Bergdoll et al (1981) and Reiser et al (1983) determined by molecular exclusion chromatography. The calculated

molecular weight by gel filtration in this study was 18.5 kDa while analysis by SDS-PAGE indicated a molecular weight of 22 kDa consistent with that reported by Schlievert et al..(1981). According to Reeves and coworkers, the discrepancy in molecular weights by the different methods is due to a combination of denaturation with a detergent and the tendency of molecular weight determination by SDS-PAGE analysis to result in overestimation. These authors feel that the lower value is more accurate. The range of molecular weights described for TSST-1 may also be a result of different purification procedures and analytical techniques.

The isoelectric point of 7.0 calculated for the toxin after resolution by isoelectric focusing in a pH gradient of 3-8 is also comparable to values reported by other investigators and reviewed by Poindexter and Schlievert, 1985.

Although it is generally accepted that TSST-1 is a simple protein, Notermans and Dufrenne (1982) found that purified TSST-1 analyzed under reducing conditions by SDS-PAGE consisted of three bands, one with a molecular weight of 23 kDa and two with molecular weights of under 13 kDa. All purified toxin preparations analyzed by SDS-PAGE in this study migrated as single band. In affinity purification of the toxin, the goat anti-TSST-1 IgG used as a ligand was prepared against TSST-1 that underwent a final purification

step on nondenaturing polyacrylamide gels. However, even the TSST-1 eluted from the goat anti-TSST-1 affinity column only showed one band when resolved by SDS-PAGE under reducing conditions. Use of goat IgG for affinity purification was desirable because of its low reactivity with protein A which is produced by many strains of S. aureus. Co-purification of protein A has the potential of interfering with TSST-1 assays where immunoglobulins from species that strongly react are used.

Production of specific antibody to purified toxin. In the attempt to produce specific anti-TSST-1 in a rabbit, polymerized toxin was used as an immunogen. This was because rabbits had been reported to have an inconsistent humoral response to inoculation with native TSST-1 (Schlievert et al.,1983). It was found by Nelson-Rampy et al. (1981) that polymerized protein of small molecular weight did not induce unresponsiveness as did the native protein. It was thought that polymerization of the toxin with ethylchloroformate would therefore enhance the immune response to TSST-1 as this technique had been used successfully to improve the immunogenicity of other small proteins (Mahfouz et al.,1980).

However, even after repeated inoculations the animal did not respond. It is possible that the rabbit was a nonresponder as previously observed (Schlievert et

al.,1983) although polymerization of the toxin should have prevented this effect. A recent paper by Poindexter and Schlievert (1987) describes the specific activation of T suppressor lymphocytes and subsequent inhibition of the antibody response of mouse spleen cells to sheep red blood cells. It is unlikely that the polymerized TSST-1 caused a suppression of the humoral response but probably the ethylchloroformate denatured the antigenic sites on the molecule. The lack of immunoreactivity to TSST-1 reported in rabbits is inconsistent with the high prevalence of anti-TSST-1 antibodies in humans colonized with toxin producing strains of staph (Ritz et al.,1984) and may indicate that rabbits respond somewhat differently to the toxin than humans.

When purified anti TSST-1 was used to immunize a goat, antibody response was rapid with high titers obtained four weeks after the initial immunization. The procedure of using separation by PAGE as a final purification step prior to immunization has been reported elsewhere (Bartel and Issel,1978) and is an effective means of preparing the antigen. It is likely that the presence of the finely ground polymerized acrylamide has an additive effect to the adjuvant and increases phagocytosis and consequent presentation of the antigen to T cells.

Relationship of tampon fibers to TSST-1 production in

vitro. When the Mg^{++} chelating ability of the Rely tampon was examined in detail it was found that carboxymethylcellulose (CMC) not polyester foam (PEF) was responsible for previously observed (Mills et al.,1985) cation binding ability. Since the CMC tended to break up and disperse throughout the Rely tampon, it was difficult to separate it completely from the PEF. Probably when PEF was tested previously for chelating ability, it contained some CMC. The CMC was most likely responsible for the observed binding of Mg^{++} (Mills et al.,1985, Schlievert, 1985). In order to examine the chelating properties of the PEF alone it was necessary to free it of CMC by exhaustive washing. PEF separated from CMC in this fashion did not bind Mg^{++} from aqueous solution or from BHI. In contrast, the CMC portion was found to bind the greatest amount of Mg^{++} per gram of fiber of any of the fibers tested. The magnesium ion binding ability that has been attributed to the Rely tampon is therefore, due to the presence of CMC not PEF.

When the chelating ability of polyacrylate rayon (PAR) was examined it was found as previously reported (Mills et al.,1985 and Schlievert et al.,1985) to avidly bind Mg^{++} from both aqueous solution and BHI. Interestingly when BHI was treated with washed PEF and PAR and then used to culture TSST-1 producing strain MN-8, cultures grown in PEF extracted BHI were comparable to the control cultures and

were not stimulated to produce more toxin. Addition of magnesium stimulated growth but not toxin production. In contrast, the medium treated with PAR did not support growth of the test organism. Addition of Mg^{++} at 10 ppm restored growth and stimulated toxin production while higher levels incrementally decreased the amount of TSST-1 produced.

When strain MN 8 was grown in BHI containing either PEF or PAR, it was found that both fibers supported growth and stimulated toxin production. The concentration of Mg^{++} was determined in culture supernatants and there was none detectable in the PAR cultures and the PEF cultures had levels comparable to the controls. This reconfirms the avid binding of magnesium by PAR and the lack of binding by PEF. As evidenced by the growth of the organism in the presence of PAR but not in fiber extracted medium, it appears that the PAR fiber bound magnesium is sufficiently available to the organisms to allow growth.

In experiments where the additive effect of magnesium on toxin production both with and without fibers was examined it appears that addition of Mg^{++} has a more direct effect on PAR cultures than PEF cultures. While additional Mg^{++} does appear to moderate the amount of toxin that is produced in the presence of PEF it does not appear to be a result of Mg^{++} chelation. On the other hand, PAR has the capacity to bind large amounts of the cation and production

of the toxin in the presence of PAR seems directly influenced by this capacity. CMC, although having the greatest capacity to bind magnesium of the fibers tested does not appear bind it in such a way that has an influence on toxin production. In other words, CMC bound Mg^{++} is readily accessible to the microorganisms.

There has been contradictory findings in the literature on the influence of tampon fibers on growth and TSST-1 production. Schlievert et al.(1984) found that addition of tampon fibers to cultures under conditions favorable for toxin production either had no effect or inhibited growth and toxin production.

Similar results were reported by Broome et al. (1982) when the interaction of tampon fiber and S. aureus were examined. They also found medium containing a polyacrylate rayon tampon failed to support growth of the organism after 4 hr and addition of defibrinated blood served to overcome this effect.

Consistent with the results of the study presented here, Reiser and colleagues (1987) did not find inhibition of growth or toxin production by any tampons including ones containing PAR. They used a procedure where tampons were moistened with buffer placed in a dialysis sac, inoculated and surrounded with molten BHI agar. The only nutrients available for growth were those diffusing in through the dialysis sac.

In a separate study from the same laboratory (Robbins et al., 1987) using a tampon disk-membrane agar procedure, all tampons tested supported growth and toxin production. The organisms grew prolifically at the membrane-tampon interface and comparable levels of toxin were produced with all types of tampon fibers tested including cotton,rayon and polyacrylate rayon. These results suggest that most in vitro protocols for assessing the influence of fiber on toxin production magnify fiber specific differences and this may not be applicable to the situation that exists in vivo.

It became apparent during the course of this study, that subtle changes in technique could dramatically impact the results particularly in respect to TSST-1 production. An example of this is where, in a preliminary experiment, the volume of medium per tube was increased from 5 ml to 10 ml. With cultures grown in the presence of cotton or PAR, this change had no effect. In the presence of PEF, however, which typically stimulated TSST-1 production to higher levels than all other fibers tested, toxin levels were no higher than control cultures without fiber.

The standard procedure used here to assess the effect of fibers on TSST-1 production consisted of 10 % fiber in a flask. At this proportion the fiber was almost completely surrounded by air and all the medium was contained within the fiber. The more absorbent fibers were barely moistened

and had to be squeezed to recover enough culture fluid for testing. An observation on the fiber cultures grown this way is that the organisms grew most prolifically at the glass fiber interface and were also much more pigmented (orange) than when grown on a streak plate. This is consistent with observations of Robbins et al. (1987) where dense growth of orange colonies was observed at a membrane-fiber interface. It seems logical to carry these observations over to in vivo and theorize that in the vagina, growth of S. aureus would occur at the mucosa-fiber interface and the organism would be present in a layer on the surface of the tampon rather than diffusing throughout the fiber matrix. This would be comparable to growth on the surface of an agar plate.

It has been well established that to produce TSST-1, cultures require an aerobic environment (Todd et al.,1987, Kass et al.,1987, Schlievert and Blomster,1983). Although the vagina is normally anaerobic, Wagner et al. (1984) demonstrated that introduction of tampons into the vagina temporarily increased the pO_2. One hypothesis on the role on tampons in the development of TSS as discussed by Poindexter and Schlievert (1985) is that they serve to oxygenate the vagina.

When the effects of aeration was compared to stationary culture (Table 7) in a system where the fiber/culture had much surface area exposed to air, shaking incubation still

increased toxin production by cultures in the presence of PEF. There was little difference observed in the PAR and cotton grown cultures under either condition. These results combined with different effects that volume had on toxin production in the presence of PAR and PEF indicate that these two fiber matrixes differ not only in Mg^{++} binding ability, but also in the way oxygen is held within the fiber. It appears that cultures in the presence of PEF under certain conditions become anaerobic rapidly, shutting down toxin production while PAR appears to allow aerobic conditions to persist for longer. It must be noted that in the whole Rely tampon, CMC may influence this tendency.

Although the relationship of tampon use to TSS has been well documented in epidemiological studies, the nature of the relationship has not been clearly elucidated. There has been marked differences reported on the effect of different fibers on TSST-1 production. There is also a great deal of variability in the techniques that have been used to examine the effect of tampon fibers on toxin production and the results are correspondingly discrepant. There are certain nutrients and conditions that influence TSST-1 production in vitro including magnesium and O_2 levels, however, there is much variation of technique where nuances of difference can radically alter the results.

Response of guinea pigs to intravaginal introduction of

fiber and TSS-associated S. aureus. The immune response of guinea pigs to intravaginal inoculation with TSS associated strains of S. aureus was monitored. When CMC and a Weck-cell surgical sponge were implanted into the animals vaginas and inoculated with TSST-1 producing and control strains of S. aureus only one out of three animals responded with a anti-TSST-1 antibodies after 6 weeks of repeated inoculation. There was also no noticeable physiological changes in any of the animals that resembled any of the diagnostic criteria for TSS. The one animal that did respond to the inoculations was immunized with strain 587 and in addition to immunoreactivity of its serum to TSST-1, it was also reactive to many other proteins produced by strain MN 8. This indicates that these two strains have production of other exoproteins in common which may be related to TSS.

When these fibers were not found to induce TSST-1 production in vitro, a second experiment was conducted using PEF which was confirmed as stimulatory to TSST-1 production in vitro. The control strain 6538-p as expected did not induce antibody production against TSST-1. These animals did have a significant fever response to the inoculation. This is not surprising since the control strain was also a pathogenic isolate and probably produced pyrogenic substances other than TSST-1.

In contrast to the in vitro data, both the cotton and PEF

samples inoculated with strain MN 8 and recovered from the animals' vaginas had comparable amounts of toxin present. This is consistent with the in vitro data of Reiser et al. (1987) where similar growth and toxin production was found in the presence of all types of tampons tested, with minimum quantitative fiber to fiber differences. The reduction in the proportion of toxin positive fiber samples recovered on successive samplings from the guinea pigs is probably indicative of a cellular infiltrate and consequent increased phagocytosis of toxin and/or bacteria. Since a function of secretory IgA is to neutralize toxin (Fukui et al.,1973) it is also possible that a specific IgA response was induced within the inoculation period and the secretory immunoglobulin acted to bind and neutralize TSST-1.

Guinea pigs tears were collected to determine if there was a specific secretory IgA antibody response to TSST-1. The production of this antibody in relationship to TSS had not been previously studied even though IgA is the primary protective antibody for mucosal surfaces (Hanson et al.,1983). Since it is known that a systemic mucosal immune response is induced when a distal mucosal surface is challenged with antigen, then a specific secretory response in the vaginal mucosa should be accompanied by stimulation of the same antibodies in the eye. The detection of specific antibodies in the eye could also prevent interference by a high level of antigen as was undoubtedly

present in the infected animals' vaginas. Although there was no specific anti-TSST-1 response detected in the tears of any animal, it does not eliminate the possibility that one was stimulated. Since the detection of secretory IgA requires specific anti-secretory piece antibodies, it is possible that the inability to detect IgA was due to a lack of specificity. Further studies to determine if there is a secretory IgA response to TSST-1 might give additional clues to the nature of protection in non-susceptible hosts.

The rapid IgG response to intravaginal inoculation observed in all animals treated with a combination of PEF and MN 8 is comparable to that which occurs by injection of an antigen. In contrast, there was no detectable humoral response in animals inoculated with cotton and the same organism even though there was comparable amounts of toxin present. Possible explanations for these results are that PEF acts as a adjuvant stimulating the humoral response, PEF disrupts vaginal mucosa and provides a portal for systemic introduction of the toxin or conversely cotton may somehow inhibit the humoral response. This last is unlikely because of the apparent similarity of cellular infiltrate in both cotton and PEF treated animals as determined by a decrease in the portion of TSST-1 positive fiber samples recovered. There was also a similar lack of a rapid response in animals inoculated with TSST-1 producing strains in the presence of CMC and weck cell

sponge. It therefore appears that PEF facilitates the transfer of TSST-1 systemically rather than cotton or CMC inhibiting it.

Conclusions. In a classic study by Elek and Conan (1957), the dramatic impact of foreign bodies on susceptibility to infection from staphylococci was studied. Where it was necessary to inject 5 x 10^6 S. aureus cfus under the skin of human subjects to produce infection, only 100 cfu were required if introduced under the skin using a silk suture. Assuming that the silk suture is an analogous to tampons introduced into the vagina and nasal or surgical packing, it appears probable that one factor in the development of TSS is protection of the causative organisms from the host's defense mechanisms. The presence of a fiber matrix under the right conditions may inhibit phagocytosis of the organisms for long enough to allow rapid proliferation and concomitant expression of toxins and extracellular enzymes.

Another contributory effect may be as indicated by the work described here is the apparent fiber specific enhancement of transportation of TSST-1 into the system as suggested by an immune response to the toxin. A third factor revealed by the low prevalence of serum antibodies to TSST-1 in TSS patients is host susceptibility. A fourth factor that is requisite for the development of toxic shock syndrome is the presence of the etiologic organism.

Although the rabbit model has been successful in reproducing many of the symptoms of toxic shock syndrome there is no animal system currently available that provides a means of assessing the potential influence that a tampon fiber can have on the development of TSS. The distinct differences in the humoral response of animals in the presence of a fiber that has a high epidemiological correlation with TSS in comparison to one with a low correlation is worthy of further study. This system may provide a means to evaluate fibers in vivo for the potential of enhancing the development of TSS in a susceptible individual.

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